Rodrigo R. Resende
Henning Ulrich *Editors*

Trends in Stem Cell Proliferation and Cancer Research

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Invited Preface

Convergent and Divergent Signaling Pathways in Stem Cells and Cancer

The rapid progress in research in areas of stem cells and cancer calls for frequent comprehensive and critical overviews of these fields. This book, presenting a compilation of the most recent reviews from these areas, is an excellent example of such needed overviews. The Editors were able to engage a large number of expert authors who present 23 chapters devoted to critical appraisal of the important topics at the forefront of the research in these fields. The collection of reviews on both the subjects, the stem cells and cancer, in a single volume, offers the reader an opportunity and convenience to learn and compare on similarities and disparities in the signaling pathways and regulatory machineries in these respective states of cell growth versus malignancy.

Of particular interest, widely covered in several chapters, is the role of stem cells in cancer genesis, development, progression, and as a potential target for cancer therapy. There is still a lot of uncertainty in identification of cancer stem cells, particularly in solid tumors, problems with their isolation, growth, and characterization of their drug sensitivity. Effectiveness of anticancer drugs on cancer stem cells cannot be properly recognized because it is still based on assessment of a decrease in tumor mass ("cancer response") where the cancer stem cells are in a minor proportion. Therefore their sensitivity may be unrelated to tumor mass shrinkage. The knowledge on identification and properties of cancer stem cells, presented in these chapters, provides the necessary background needed for further advances towards development of more effective anticancer strategies.

Among many attention-grabbing chapters the article describing embryonic rest hypothesis of cancer development is especially exciting. Based on evidence of the presence of very small embryonic/epiblast-like stem cells (VSELs) in different tissues the authors of this chapter resurrect the 150-year-old hypothesis of Rudolf Virchow and Julius Cohnheim who proposed that cancer may develop from embryonic cell remnants that remain in the developing organs following embryogenesis ("embryonic rest hypothesis of cancer development"). Their contention that the primitive epiblast-germline-derived VSELs represent the Virchow's

hypothetical embryonic cells from which cancer develops is exciting, calls for further experiments testing, and if confirmed, opens new avenues for cancer prevention and possibly the treatment.

Several chapters are devoted to anticancer strategies aimed toward selective killing cancer—while sparing normal—cells. Some strategies are focused on modulation of the mechanism of cell death by the mode of apoptosis, autophagy, and other means of cell demise. Among these chapters very exhaustive and stimulating is the review describing the role of BH3-only proteins, a class of small molecules of the Bcl-2 family, in regulation of cell propensity to undergo apoptosis. In this chapter the authors present attractive ideas of how to exploit mechanisms involving BH3-only proteins for selective elimination of cancer cell, offering one more cancer treatment strategy.

Cancer can be considered as primarily a disease of the cell cycle. While normal cells enter into—and progress through—the cell cycle only when triggered by the respective growth factors or mitogens, cancer cells are constitutively recruited to the cycle by the persistent stimulation of many signaling pathways mobilized by the defective oncogenes and/or tumor suppressor genes. A number of chapters address these differences and discuss the cell cycle-oriented anticancer strategies. The last chapter (23) of this volume specifically addresses targeting the cell cycle for anticancer treatment. It offers a very comprehensive review of the cell cycle regulatory machinery, describes the cancer-related changes in this machinery, and lists pharmaceutical agents targeting individual constituents of the machinery and the associated signaling pathways.

All in all, this book is a valuable compendium covering the most important topics related to cancer and stem cells and presenting comprehensive and state-of-the-art reviews. It can be of assistance to researchers who are already deeply engaged in the respective fields in cancer and stem cell biology. It can be of even greater assistance to newcomers to these fields giving them a possibility to see the diversity of the subjects and also, through the depth of the field covered in individual reviews, the outlook of entirety of the up-to-date research progress along these subjects. Because several chapters contain novel ideas on anticancer strategies as well as on a potential role of stem cells in regenerative medicine, the book will intellectually stimulate readers in these directions.

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Editor Preface

More and more scientists and physicians are being confronted with the advancement in stem and cancer cell research and mechanisms of proliferation and cell survival. Throughout life, complex genetic systems regulate the balance between cell birth and death in response to growth and death signals. In this dynamic process, stem cells are essential for development, tissue repair, as well as aging and cancer. Major importance is given to mechanisms resulting from genetic and epigenetic alterations that result in changes of pathways mediating normal cellular activities.

This book provides comprehensive coverage of the current state-of-science of molecular stem and cancer research with discussion of controversial issues, directed to a wide range of audience including scientists working in the field, physicians, and all others who need to understand the molecular mechanisms that govern the functioning of stem cells in normal physiology and malfunctioning of tumor cells.

Experts in the field contributed 23 chapters, grouped into four parts. The first part "*Molecular Bases of Stem and Cancer Cell Proliferation*" focuses on mechanisms of proliferation control, initiated by an introductory chapter and followed by a chapter on the participation of extracellular matrix in the establishment of stem and cancer cells and the embryonic hypothesis of cancer development, suggesting that tumor genesis occurs from remaining stem cells as a development-related process. Furthermore, maintenance of pluripotency and proliferation processes are strictly regulated by intracellular pathways events. Induction of intracellular calcium transients as well as sphingolipid formation and lipid droplet biogenesis acting as inducible organelles play an important roles in cell signaling.

The part "*Signaling Pathways Underlying Tumor Genesis, Proliferation and Cell Death*" aims to highlight signaling processes and pro-and anti-apoptic mechanisms which decide over proliferation or cell death. A chapter discusses the breakthrough immunomodulatory mechanisms of CL-B and its importance in antitumor responses. The importance of BH3-only proteins in regulation apoptosis is described in addition to the participation of β-catenin/JNK and NF-KB signaling in carcinogenesis and cell death.

The third part focuses on "*Functions and Dysfunctions of Physiological Processes Involved in Tumor Genesis and Malignancy*" including the role of

chemokines, autophagy, epigenetic features, and immune response mechanisms exerted by tumor-associated neutrophils with implications in tumor development and malignancy. Mesenchymal stem–cancer stem interactions as well as regulatory functions exerted by microRNAs provide further mechanisms and possible targets for combating tumor cells. The final part "*Strategies for Cancer Treatment*" includes a contribution on cancer stem cell markers for characterization of the tumor and prognosis of patient treatment and survival. Furthermore, cancer cell dormancy and cell cycle regulation provide molecular targets for anticancer therapy.

In summary, together with aspects of stem cell biology, this book puts together knowledge on various molecular aspects of cancer, the disease with the highest predicted mortality in the twenty-first century, and encourages physicians and researchers from various disciplines to join their experience and efforts in combating this disease.

Belo Horizonte, November 2012 Rodrigo R. Resende
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Part I
Molecular Bases of Stem and Cancer Cell Proliferation

Chapter 1
An Introduction to Proliferation and Migration of Stem and Cancer Cells

Micheli Mainardi Pillat, Talita Glaser, Telma Tiemi Schwindt and Henning Ulrich

Abstract Throughout life, complex genetic systems regulate the balance between cell birth and death in response to growth and death signals. During early development, only a few cells abandon the cycle, but in several adult tissues, the cells normally do not proliferate, except during healing processes, which are supported by stem cells. However, in some adult tissues, cells continuously divide as a strategy for constant tissue renewal. In this context, cancer occurs when the control of growth and death is defective, driving the cells to an erroneous escape from death and causing intense cell proliferation. In the same way, the mechanisms and processes that coordinate cell migration are related to cell–cell contact and are important for homeostasis and the constitution of the organism. Moreover, migration is a normal event during embryo development and tissue regeneration; however, when regulation of migration fails, this can lead to a diverse number of diseases, including cancer. This chapter introduces the reader to the following specialized chapters on proliferation mechanisms written by experts in the field.

Keywords Stem cells • Cell proliferation • Cancer • Migration

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1.1 Proliferation

1.1.1 Cell Cycle

Cell cycle control must be extremely accurate, because it manages one of the most important processes in nature, the birth of a new cell. Its understanding is so important that the investigators who conducted the initial experiments elucidating the main regulators of the cell cycle in eukaryotes, Leland H. Hartwell, Tim Hunt, and Sir Paul Nurse, were awarded with the Nobel Prize in Physiology or Medicine in 2001. Basically, the cell cycle is divided into *four phases* (shown in Fig. 1.1). Two of these phases are responsible for the execution of the basic events in cell division: duplication of the genetic material (the synthetic phase or S phase) and partition of the cellular components into two identical daughter cells (mitosis or M phase). The other two phases of the cycle are gap periods (G1 and G2 phases), when cells prepare themselves for the subsequent initiation of the S and M phases. Cells may exit the cycle and stay in a nondividing state known as a quiescent state, called G0. Stem cells are often found in the G0 state.

The passage from one phase to the next is regulated by *cyclins* and *cyclin*-dependent kinases (CDKs; Fig. 1.1). Cyclins are proteins synthesized and degraded throughout the cell cycle at specific checkpoints. They gain their regulatory role by activating CDKs. On the other hand, CDKs are serine/threonine kinases that form active heterodimeric complexes after binding to cyclins. Several cyclins and CDKs are involved in cell cycle control: three interphase CDKs (CDK2, CDK4, and

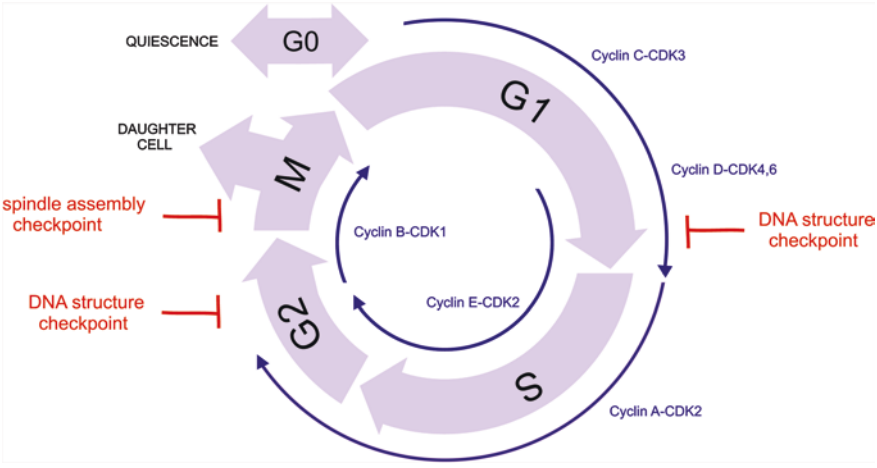

Fig. 1.1 Cell cycle: phases, cyclins, cyclin-dependent kinase (*CDK*) activities, and checkpoints. Phases of the cell cycle: *G1* gap 1 phase, *S* synthesis phase (period of DNA replication), *G2* gap 2 phase, *M* mitosis phase (period of chromosome separation and cytokinesis), *G0* resting phase. Cyclin–CDK activities during individual phases. Three major checkpoints are shown

CDK6); one mitotic CDK (CDK1, also known as cell division control protein 2 or Cdc2); ten cyclins belonging to four different classes (the A, B, D, and E types). The cyclin–CDK complexes regulate the activities of several proteins involved in DNA replication and mitosis by phosphorylating them, activating some and inhibiting others in a coordinated manner. It is also important to emphasize that tumor-associated mutations frequently deregulate some cyclin–CDK complexes (Morgan 1997; Malumbres and Barbacid 2009; Deckbar et al. 2011).

The high fidelity of cell division is due to *checkpoint* surveillance mechanisms (Fig. 1.1). It ensures that one phase of the cell cycle is not initiated until the previous process has been successfully completed. There are checkpoints at S and M phase initiation and during the M phase (anaphase, telophase, and cytokinesis onset). DNA damage, for example, activates checkpoint mechanisms by targeting the cyclin–CDK complexes, thus interrupting cell cycle progression to provide extra time for damage removal. Mitotic spindle checkpoints, for example, are activated indirectly by sensing the consequences of the damage, such as incorrect alignment at the equatorial plane or impaired formation of the spindle fibers (Hartwell and Weinert 1989; Malumbres and Barbacid 2001; Deckbar et al. 2011).

All cells are able to enter the *quiescent* state and to return to the cycle, except those in terminal differentiation, depending on extracellular and intracellular scenarios that control this important decision. Quiescent and newly divided cells probably must also pass certain checkpoints before they return to the cell cycle, such as the verification of the homeostatic size of the cell and the influence of the size of the tissue. These parameters are mainly regulated by extracellular signals, such as the availability of nutrients and the intensity of growth and mitogenic stimuli. Conversely, cancer cells lose normal control in replication and can proliferate in conditions in which a normal cell could not, for example, in the presence of DNA damage and in the absence of mitogenic stimuli.

1.1.2 Cell Division Signaling in Cancer

Tumors display mutations in genes encoding proteins involved in the regulation of proliferation, survival, and apoptosis. To emphasize the role of these genes in cancer development, they are termed proto-oncogenes or tumor suppressor genes, two broad classes of genes implicated in carcinogenesis (Table 1.1). Proteins encoded by *proto-oncogenes* are components of intracellular networks involved in survival, proliferative, and antiapoptotic processes. Proto-oncogenes change to oncogenes by mutations that generate hyperactive and control-resistant proteins. On the other hand, proteins encoded by *tumor suppressor genes* have antiproliferative, proapoptotic, and prodifferentiation roles. In this case, carcinogenesis and tumor growth can only occur if a mutation in a tumor suppressor gene occurs, leading to inactive forms of the protein. BMI-1 (B lymphoma Mo-MLV insertion region—it belongs to the polycomb group of epigenetic chromatin modifiers), for example, is encoded by a proto-oncogene, whose expression is deregulated in certain types of cancer (Haupt

Table 1.1 Examples of proto-oncogenes and tumor suppressor genes. Mutations in these genes may cause cancer

Proto-oncogenes	Tumor suppressor genes
Erb2 (EGF receptor)	PTEN (PIP-3 phosphatase)
Ras (small G protein)	Rb (retinoblastoma tumor suppressor protein)
Akt (Ser/Thr kinase)	APC (inhibitor of β-catenin signaling)
Src (Tyr kinase)	ARF (indirect inhibitor of cell cycle)
SKP2 (S-phase kinase-associated protein 2)	p53 (transcription factor)
Jun/Fos (formation of the AP-1 early response transcription factor)	p16 (cyclin-dependent kinase inhibitor 2A)
Myc (transcription factor)	
BMI-1 (member of the polycomb group of epigenetic chromatin modifiers)	

EGF epidermal growth factor, *PIP-3* phosphatidylinositol 3,4,5-trisphosphate, *PTEN* phosphatase and tensin homolog, *APC* adenomatous polyposis coli

et al. 1991; Cui et al. 2007). BMI-1 is required for the proliferation of leukemia-initiating cancer stem cells (CSCs), and it is also highly expressed in cancer neural stem cells and in medulloblastomas arising from cerebellar granule precursor cells (Lessard and Sauvageau 2003; Leung et al. 2004; Grinstein and Wernet 2007).

1.1.3 Stem Cell Proliferation and Cancer Induction

Adult stem cells are important for tissue homeostasis and regeneration. They have the ability to self-renew indefinitely and to give rise to transit amplifying cells, which are capable of differentiating into tissue-specific cells (Reya et al. 2001). Stem cells are usually quiescent, and this behavior has an important role in protecting them from exhausting their proliferative capacity, and in decreasing mutations that occur during DNA synthesis (Sang et al. 2008; Moore and Lyle 2011). However, stem cells can quickly proliferate in response to stressors. Hematopoietic stem cells, for example, proliferate in response to chemotherapy or bone marrow transplantation in order to rapidly produce progenitors, differentiated cells, and additional stem cells, and then return to the quiescent state (Dixon and Rosendaal 1981).

Proliferation of stem cells is highly controlled to prevent cancer development, since they have the capacity to accumulate mutations over years or decades. It is expected that, as occurs with normal stem cells, CSCs generate oncogenic transit amplifying cells, being capable of expanding tumor mass and giving rise to heterogeneous tumor populations (Reya et al. 2001; Moore and Lyle 2011). CSCs were discovered in acute myelogenous leukemia (Bonnet and Dick 1997) and,

nowadays, proof of their existence has been provided for other neoplasias, such as breast, prostate, and colon tumors and glioblastomas (Al-Hajj et al. 2003; Singh et al. 2003; Collins et al. 2005). Quiescent CSCs are important targets for cancer therapy since they are usually resistant to chemotherapy, owing to their state of dormancy. In other words, CSCs do not die during continued therapy and contribute to cancer recurrence (Dick 2008; Li and Bhatia 2011). Expanding knowledge of the mechanisms of stem cell quiescence and proliferation is important not only for understanding normal stem cell function, but also for developing therapeutic approaches to exterminate quiescent CSCs.

1.2 Cell Migration

1.2.1 Mechanisms

Besides connective tissues, cells are tied together by cell–cell contact, where cytoskeletal filaments are anchored, transmitting stresses across the interior of the cells. There are two types of cell contact: adherens junctions, which anchor actin filaments, and desmosome junctions, which attach to intermediate filaments. Transmembrane adhesion proteins link cytoskeleton to extracellular structures, and are divided into two superfamilies: cadherins, which attach cell to cell; and integrins, which attach cells to the matrix.

Cells must acquire a spatial asymmetry to enable them to migrate. First, there is a change to a polarized morphology owing to spatial or temporal stimulus gradients caused by microscopic nonuniformities or by kinetic fluctuations in receptor–ligand binding, followed by a forward redistribution of chemosensory signaling receptors (Sullivan et al. 1984), integrin adhesion receptors (Maxfield 1993), and integrin cytoskeleton linkages.

During migration, protrusion of lamellipodia or filopodia membranes requires actin-polymerization-generated force (Fig. 1.2), from the Brownian ratchet mechanism and/or the cortical expansion mechanism. Once the membrane protrusion has become adherent to the substrate, forward translocation of the cell body may occur by interaction of myosin with actin filaments, such as contraction of filaments connecting cell–substratum adhesion complexes with intracellular structures, or relative movement of adhesion complexes across cortical actin filament tracks. In both cases, the magnitude of the traction is greater than the rearward pull on the adhesion complexes. Detachment of the cell rear involves disruption of cell–substratum attachments, accelerated by myosin-mediated actin filament contraction pulling on adhesion complexes. Subsequently, the magnitude of traction is less than the contraction force in the rear.

Importantly, spatial concentrations, or localized temporal concentration transients of second messengers, such as calcium and phosphoinositides, or even enzymes and motor proteins, are distributed differentially across the cell length. As one simple regulation mechanism, high calcium concentrations at the rear of the cell

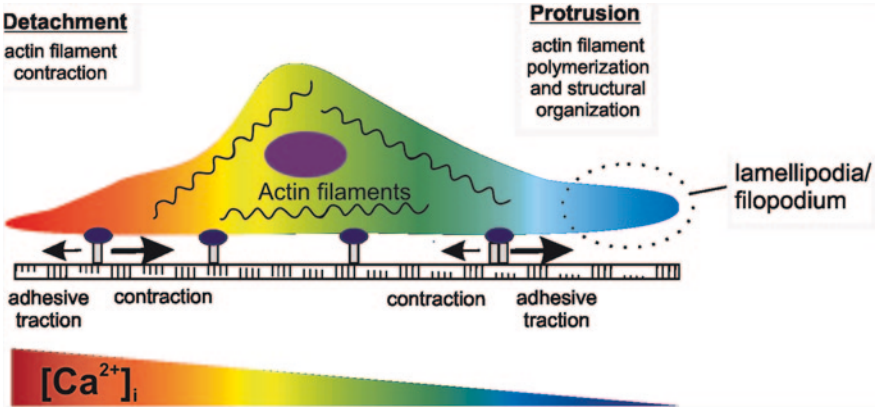

Fig. 1.2 Basic mechanism of cell migration. The actin-polymerization-dependent protrusion and firm attachment of lamellipodium. Contraction at the rear of the actin propels the body of the cell forward to relax some of the tension (traction). High intracellular calcium concentration ($[Ca^{2+}]i$) at the rear of the cell contributes to the migration process

activate proteins that disrupt actin filament networks (Janmey 1994) and enhance myosin II contractile activity to promote the release of attachments there (Conrad et al. 1993; Maxfield 1993). Low calcium concentrations and high phosphoinositide levels at the front of the cell will activate proteins that cross-link actin filaments, facilitating membrane extension (Janmey 1994); myosin I activity at the front of the cell (Conrad et al. 1993) might additionally permit directed membrane-protein transport activity to promote formation of new attachments, or contraction to pull cell body structures forward (Sheetz 1994; Lauffenburger and Horwitz 1996).

1.2.2 Epithelial–Mesenchymal Transition

Epithelial–mesenchymal transition (EMT) is the process that allows the derivation of a multitude of functionally specialized cells, tissues, and organs from an initial small variety of pluripotent stem cells present in the developing embryo. EMT is most commonly observed in the wound healing response and in angiogenesis. When epithelial cells in tissue culture undergo EMT, phenotypic alterations include the loss of cell–cell contact and the change in expression or function of proteins involved in cell–cell adhesion, given that, in development, one of the primary functions of EMTs is to facilitate cell dispersion. Cell–cell adhesion is mediated by the cadherin–catenin-based adherens junctions keeping adjacent epithelial cells together. Adherens junctions also keep the cells of the epithelia in a nonmotile and nonproliferative state. They comprise a transmembrane-spanning receptor, E-cadherin, that binds to another E-cadherin molecule in adjacent cells in a homophilic interaction. The intracellular domain of E-cadherin is linked to the

actin cytoskeleton. E-cadherin levels on the plasma membrane can be regulated by epigenetic, transcriptional, and posttranslational mechanisms (Khew-Goodall and Wadham 2005).

Posttranslational mechanisms shown to regulate cell–cell adhesion include tyrosine phosphorylation and relocalization of E-cadherin away from the plasma membrane. Moreover, tyrosine phosphorylation of cadherins or catenins leads to dissociation of the adherens junctions and thus to a decrease of cell–cell adhesion (Ayalon and Geiger 1997; Ozawa and Kemler 1998a, b; Roura et al. 1999). Therefore, regulation by phosphorylation is expected to be highly reversible and to lead to a transient loss of cell–cell adhesion, unless secondary events occur that either prevent dephosphorylation or increase turnover of the phosphorylated proteins.

A number of transcription factors capable of repressing E-cadherin transcription, namely, Snail (Batlle et al. 2000), Slug (Bolos et al. 2003), ZEB1 (Grooteclaes and Frisch 2000), and ZEB2 (Comijn et al. 2001), have been found to be upregulated during EMT. These transcription factors can repress certain epithelial genes such as E-cadherin and cytokeratin-8 and increase the expression of mesenchymal genes. Furthermore, Snail(-/-) mice fail to complete gastrulation because of a defective EMT (Carver et al. 2001). The dual role of Snail, to shut down expression of the epithelial genes and to turn on expression of mesenchymal genes, leads to the idea that it could be an EMT inducer.

1.2.3 Stem Cell Migration and Cancer

The cancer environment comprises tumor cells and a wide network of stromal and vascular cells participating in the cellular and molecular events necessary for invasion and metastasis. Tumor secretory factors can activate the migration of host cells, both near to and far from the primary tumor site, as well as promote the exodus of cells to distant tissues. Thus, the migration of stromal cells and tumor cells among specialized microenvironments occurs throughout tumor and metastatic progression, providing evidence for the systemic nature of a malignancy.

Mesenchymal stem cells (MSCs) are recruited from bone marrow to areas of inflammation or damage by local endocrine signals, resulting in the formation of fibrous scars. Tumor tissue contains abundant growth factors, cytokines, and matrix-remodeling proteins, explaining why tumors are linked to wounds that never heal. MSCs are reported to migrate to injury or tumor sites and to incorporate themselves into tumor stroma, but the effects of the interactions between MSCs and tumor cells, as well as the mechanisms underlying these effects, remain unclear. Recent experiments revealed that MSCs promote tumor growth and metastasis. Reports suggest that MSCs are involved in tumor invasion and angiogenesis, immunosuppression, and inhibition of apoptosis. Moreover, some studies reported that MSCs can differentiate into carcinoma-associated-fibroblast-like cells by prolonged exposure to tumor-conditioned medium and that these cells promote tumor growth (Shinagawa et al. 2010).

EMT in the adult is now recognized to be the forerunner to a number of pathological states. In the progression of epithelial tumors to metastatic disease, EMTs give rise to a cell type that is beyond recognition as an epithelial cell and confers on it the ability to proliferate and invade the basement membrane and surrounding stroma. Epigenetic mechanisms include methylation of the E-cadherin gene, which has been observed in a number of human cancers (Khew-Goodall and Wadham 2005).

Ovarian cancer is the most lethal of all gynecological malignancies, and the identification of novel prognostic and therapeutic targets for ovarian cancer is crucial. It is believed that only a small subset of cancer cells is endowed with stem cell properties, which are responsible for tumor growth, metastatic progression, and recurrence. NANOG is one of the key transcription factors essential for maintaining self-renewal and pluripotency in stem cells. Siu et al. (2012) demonstrated that NANOG was highly expressed in ovarian cancer cell lines with metastasis-associated property and in clinical samples of metastatic foci. Stable knockdown of NANOG expression prevented ovarian cancer cell proliferation, migration, and invasion, which was accompanied by an increase in messenger RNA expression of E-cadherin, caveolin-1, FOXO1, FOXO3a, FOXJ1, and FOXB1. Conversely, ectopic NANOG overexpression enhanced ovarian cancer cell migration and invasion along with decreased E-cadherin, caveolin-1, FOXO1, FOXO3a, FOXJ1, and FOXB1 messenger RNA expression. Importantly, NANOG mediates cell migration and invasion and is involved in the regulation of E-cadherin and FOXJ1 expression.

The identification of the restrictive mechanisms that prevent the triggering of cellular transitions in adult organisms in cancerous tissues may lead to the development of tools for therapeutic tissue repair and effective tumor suppression (Prindull and Zipori 2004).

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Chapter 2
The Role of Cell Adhesion, Cell Junctions, and Extracellular Matrix in Development and Carcinogenesis

Anderson K. Santos, Fernanda M. P. Tonelli, Daniel A. F. Silva, Kátia N. Gomes, Luiz O. Ladeira and Rodrigo R. Resende

Abstract Cells express different cell adhesion molecules (CAMs) which guarantee anchorage, polarity, and support for cells. However, CAMs do not only act mechanically as contact sites between the cell and the extracellular matrix or neighboring cells, but also trigger signaling pathways, including survival and proliferation. In this chapter, we discuss the molecular basis of CAMs and cell junctions, the effects of cell–extracellular matrix and cell–cell adhesion on normal cell survival, and mechanisms of invasion and metastasis formation during cancer development. The study of normal and pathological processes specifically related to the role of cell junctions may provide novel targets for cancer therapy.

Keywords Cell junctions • Cell adhesion molecule • Claudins and occludins • Immunoglobulin-like cell adhesion molecule • Cadherins • Selectins • Integrins • Connexons • Extracellular matrix • Cytoeskeleton • Signaling pathways • Cancer progression • Metastasis • Cancer invasion

2.1 Introduction

What keeps cells bound together in our bodies? Is there communication between these cells? Proliferation, signaling transduction, structure modeling, and differentiation are processes guided by molecules on the outer cellular membrane and guarantee the stability and development of multicellular complex structures.

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There is a molecular apparatus known as the extracellular matrix (ECM) composed of proteins, glycoproteins, glycolipids, and complex polysaccharides secreted by cells. The composition of the ECM defines the strength of interactions between itself and cells. The cytoskeleton and cell–cell adhesion also have an important role in cell activities, as will be seen later in this chapter. In animals, there are a variety of tissues which are greatly specialized by differentiation; thus, they are classified through generic architectural structure: connective and epithelial tissue. In connective tissue, the ECM is abundant to support a high mechanical tension and not to transmit this pressure to cells (cartilage and bones). In epithelial tissue (e.g., epidermis and intestinal mucosa), cells are tightly attached, forming the epithelium. The ECM in this tissue is scarce, corresponding to a thin layer known as the basal lamina. Cells bind to each other by cell–cell adhesion, where they are anchored through cytoskeleton filaments which transmit the stress suffered (Miyoshi and Takai 2005; Herve et al. 2007; Hartsock and Nelson 2008).

The properties which allow cell–cell and cell–ECM contacts differ by cell type and function because of the specificity and specialty of the tissue, beyond cell polarity. The junctions formed are distinct in structure and, consequently, transmit its physical strength. On the basis of molecular composition, there are four types of junctions (Fig. 2.1):

1. Anchoring junctions: cell–cell and cell–ECM adhesions linked to cytoskeleton filaments that distribute the strength when the cells are physically stressed
2. Communication junctions: protein channels linking different cytoplasms, allowing the flow of small molecules and ions
3. Occluding junctions (tight junctions): junctions that join the membranes of two adjacent cells and seal the spaces between cells, creating an impermeable or a selectively permeable barrier to fluid

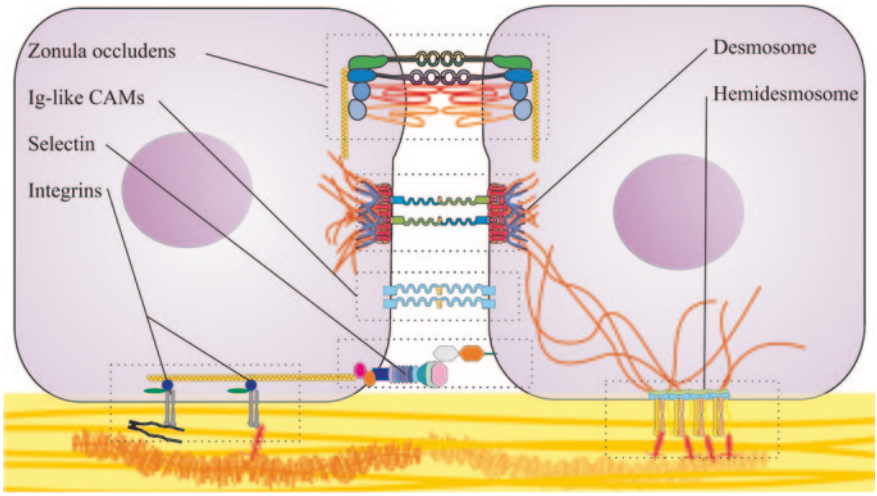

Fig. 2.1 Extracellular matrix (ECM) overview: functional classes of cell junctions in animal tissue. *CAMs* cell adhesion molecules

4. Signaling junctions: junctions that allow signaling molecules to flow from a cell to the membrane of adjacent cells in places where there is cell–cell contact (e.g., chemical synapses in the nervous system)

In the first part of the chapter, we will discuss the role of each type of protein involved in the junctions mentioned above in the development of animal tissues, and then the junction structures.

Some animal diseases are caused by alterations in the ECM. Cancer, for example, is a process that occurs when a unique cell mutates and cannot control its division and expansion, and does not respond to external stimuli from the ECM (Ben-Zion 2010). The nature of cancer still needs to be elucidated in detail. Some molecular alterations are able to turn a normal cell into a cancerous one, which acts individually, dividing like a germinative cell. At any time, the tumor cell can lose contact with the basal lamina and adjacent cells and becomes able to invade other tissues, a process known as metastasis (Zhong and Rescorla 2012).

There are alterations in expression of adhesion proteins that cause loss of contact, in addition to expression of proteins which degrade the ECM and allow cell invasion (metastasis). In the second part of this chapter, we will discuss the main alterations in the behavior of ECM components and in the cytoskeleton which lead to establishment and progression of cancer. Cell junctions seem to have an important role in cancer progression and metastasis, and, therefore, knowledge of underlying mechanisms may provide novel treatment options (Veiseh et al. 2011).

In this chapter, major components of cell adhesion, junctions, and the ECM, beyond a brief review of the role of these components in cancer progress and metastasis, will be discussed.

2.2 Extracellular Matrix

The ECM is composed of different families of proteins, glycoproteins, and complex polysaccharides, each performing a different function in the ECM. For example, integrins mediate cell–ECM binding, whereas cadherins have an important role in cell–cell binding (Gomez et al. 2011). Each family is specialized: cadherins link to intermediate filaments to form desmosomes in the same way integrins can link to actins to form adherens junctions or promote cell–ECM adhesions or can link to intermediate filaments to form hemidesmosomes. There are exceptions in proteins linkage, probably because of the different types of cadherins and integrins (Fig. 2.2).

2.2.1 Glycosaminoglycans

What are glycosaminoglycans (GAGs)? GAGs are one of the ECM's components and have a linear polysaccharide structure, composed of disaccharide units consisting of

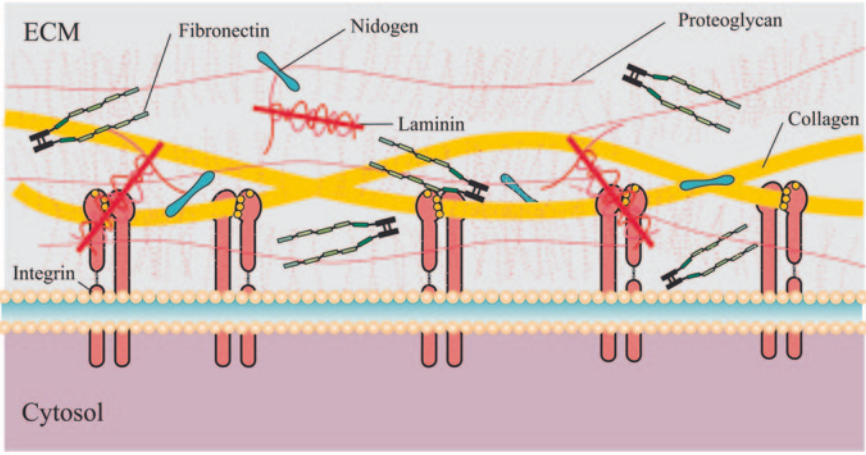

Fig. 2.2 Composition of the ECM. The ECM influences survival, development, morphology, motility, and polarity. Its molecular composition differs between tissues, together with the expression levels of the compounds. The ECM contains several fibrous proteins in a medium with high concentrations of glycosaminoglycans and proteoglycans. Fibrous proteins such as collagens (of several types) anchored in proteins such as integrins, fibronectins, laminins, and glycoproteins make cells resistant against mechanical stress

an amino sugar (e.g., *N*-acetylglucosamine) and a uronic acid (e.g., glucuronic acid) or galactose (Esko et al. 2009). Sulfate groups are commonly present in sugars. Together with carboxyl functions, these groups can confer a total negative charge to GAGs.

GAGs are highly hydrophilic and participate in the establishment of resistance to local mechanical forces. These molecules also play a role in the regulation of diffusion of small molecules and ions through the cell membrane. Thus, ECM GAGs can adopt an extended conformation, bind water molecules, and attract cations.

According to their sugar composition, GAGs can be classified as heparan sulfate, keratin sulfate, dermatan sulfate, and chondroitin sulfate, and can be found either on the cell surface or free in the ECM. The simplest GAG is hyaluronic acid, which has no modifications (Bülow and Hobert 2006).

Besides being essential for the ECM's form, in vitro GAGs were able to regulate cancer cell adhesion and growth; heparan sulfate can induce fibroblast growth factor dimerization and dermatan sulfates can modulate the action of vascular endothelial growth factor (Berry 2005).

Hyaluronan (or hyaluronic acid) is abundant in connective tissues, and the structure of this linear polysaccharide is recognized by some proteins capable of binding it to proteoglycans, stabilizing the matrix structure. On articulation, hyaluronic acid acts as a lubricant, and during embryogenesis it can fill empty spaces and make it easier for cells to migrate from one location to another, which also occurs during the wound healing process (Fraser et al. 1997; Laurent et al. 1995, 1996).

2.2.2 Proteoglycans

A proteoglycan is one or more GAG chain covalently attached to a protein core. Proteoglycans are a heterogeneous group of glycoconjugates. Like GAGs, proteoglycans are negatively charged and ubiquitous in animals. Once they present structural diversity, proteoglycans also have diverse functions besides being part of a natural scaffold (Aumailley et al. 2000a; Gesteira et al. 2011). Proteoglycans can also participate in tumor growth. Decorin, an ECM proteoglycan, can control inflammation and tumor growth; acting as a ligand for Toll-like receptors 2 and 4 and stimulating the production of proinflammatory molecules, it can suppress tumor growth (Merline et al. 2011).

Proteoglycans also can regulate the activity of secreted proteins (e.g., by binding them). During neuronal differentiation, heparan sulfate proteoglycans modulate signaling pathways in axon guidance, and chondroitin sulfate proteoglycans modify the guidance function of semaphorins (de Wit and Verhaagen 2007). Proteoglycans form cell surface receptors. On epithelial cells, they act as receptors for interstitial matrix components, and heparan sulfate proteoglycans can be a viral receptor as well (Hunter 1991).

2.2.3 Collagen

Collagen is the most abundant protein in mammals, accounting for 25 % of the total protein. It also acts as a structural scaffold on the ECM of tissues. The collagen family of proteins have a stiff structure formed by triple-stranded helices. Ninety percent of all collagen in the human body can be found in connective tissues (collagen types I, II, and III) (Brett 2008).

Collagen is divided into three groups: fibrillar collagens, for example, types I, II, III, and V; fibril-associated collagens, for example, types IV and IX; sheet-forming collagens, for example, type IV. There are at least 16 types of collagen, the major insoluble fibrous proteins in the ECM (Lodish et al. 2000).

Each collagen polypeptide chain is called an α chain. It is carefully organized in triplets, and the commonest is proline–hydroxyproline–glycine. During biosynthesis of collagen I, for example, after pro-α-chain synthesis (protocollagen strand), prolines and lysines are hydroxylated; three arginine to cysteine substitutions during the translation process cause Ehlers–Danlos syndrome, an increasing propensity for arterial rupture in early adulthood. Then, after removal of propeptides, some hydroxylysines are glycosylated and three α chains combine to form procollagen. Following processing of procollagen by proteinases N and C, cross-links between lysine and hydroxylysine residues are established by lysyl oxidase, endowing mature collagen fibrils with stability and strength (Brett 2008; Timpl et al. 2008).

2.2.4 Fibronectin

Fibronectin, another ECM component, is a large glycoprotein that is able to promote adhesion through specific domains: cellular adhesion to other cells or to basement membrane, coagulation with clot stabilization, nerve regeneration, fibroblast migration and macrophage function, pathogen binding to cells or the ECM, and cell adhesion during embryogenesis (Proctor 1987). Mice that do not express fibronectin die because their vascular system cannot develop correctly (Astrof et al. 2007).

These molecules are organized in disulfide-bound dimers that can bind, for example, to integrin on the cell surface through the RGD motif. Each monomer of the dimer consists of a repetition of three units called fibronectin types I, II, and III (Mosher and Furcht 1981; Singh et al. 2010).

Fibronectin found on connective tissues and associated with the basal lamina is considered the insoluble form, which interacts with collagen and GAGs, for example. The soluble (nonfibrilar) form is found in body fluids such as blood and interacts with fibrinogen and fibrin, for example, and can act as an opsonin (Mosher and Furcht 1981).

2.2.5 Basal Lamina

The basal lamina or basement membrane is a type of ECM that consists of a very thin and flexible layer and supports the epithelium and also wraps muscle, adipose tissues, and Schwann cells. Besides its structural role (contributing to tissue organization, cell support, and organization of cells in monolayers during tissue development), the basal lamina can also act as a filter, such as in renal glomeruli, where it is located between two cell layers (Sanes 2003).

The basal lamina also has functions: sequestering growth factors that can affect polarity and behavior during lamina remodeling; guiding cellular differentiation, proliferation, and migration by inhibiting or promoting these processes; participating in the determination of acetylcholine receptor localization in neuromuscular synapses.

There is high variation in the composition of the basal lamina, differing from one tissue to another. However, the basal lamina mostly contains at least one isoform of each of the laminin, collagen IV, and nidogen families and proteoglycans such as perlecan (Aumailley et al. 2000b) (Fig. 2.3).

Laminin is the major matrix component and possesses structural motifs responsible for interacting with cell surface proteins, for example; that is why these cell adhesive peptides from laminins are being studied as potential candidates for cell-binding activities to form scaffolds for tissue engineering. Laminin is a cruciform-shaped protein composed by three chains: α, β, and γ, encoded by separate genes.

Collagen IV confers tensor strength to basement matrix and can interact with other collagen IV molecules, forming sheets, or with other constituents such as nidogen and proteoglycans. These binding sites are located in the interrupted surface of the collagen IV triple helix.

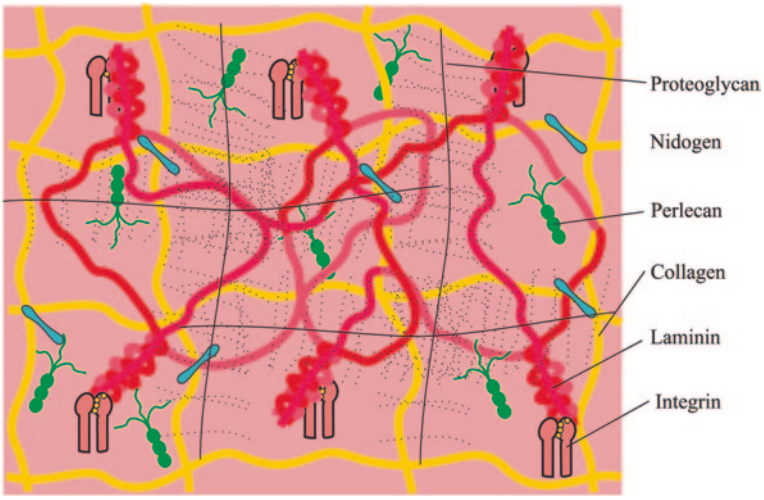

Fig. 2.3 Basal lamina

Perlecan is a large multidomain heparan sulfate proteoglycan which is also essential for the epithelium structure; in recent work, Inomata et al. (2012) showed how important this molecule is for the corneal epithelial structure; in humans, its gene contains 97 exons encoding a 467-kDa protein core with five distinct domains.

The nidogen family has two members present in the mammalian basal lamina: nidogen 1 and nidogen 2. Isoform 2 exhibits tissue specificity in adulthood, whereas both isoforms are ubiquitous and bind with similar affinities to laminin in vitro.

The minor components of the basal lamina confer tissue-specific properties to this ECM. They include, for example, agrin (heparan sulfate proteoglycan), type XV and type XVIII collagens, and secreted protein acidic and rich in cysteine (SPARC) (LeBleu et al. 2007).

2.2.6 Orientation of the ECM

Considering that the ECM is produced by cells, one can assume the existence of a close relation between them. Cells participate not only in matrix production, but also in its organization; and matrix can take part in the determination of cell surface display. The ECM and the cytoskeleton are connected by transmembrane proteins (Théry et al. 2005).

In chicken optic tectum, for instance, the matrix guides the formation of optic fibers. In vitro, the orientation of the ECM can modulate cardiac myocyte phenotype through morphogenetic information detected by the cell and translated to a phenotypic fate of differentiation. It is also proposed that the positioning of the division axis is determined by the ECM by controlling the location of actin at the membrane and thus the segregation of cell constituents.

During the bone remodeling process, the formation of the ECM, which is highly oriented and contains a large amount of collagen, requires an alignment of osteoblasts. Then, the matrix formed acts as substrate for alignment of other osteoblasts as the ECM is built (Etienne-Manneville 2011; Kerschnitzki et al. 2011).

Thus, the organization of the ECM influences cell organization and they both influence tissue functionality. Understanding these interactions is essential for the advance of tissue engineering and the production of optimized 3D scaffolds to be used for clinical purposes (Guillemette et al. 2009).

2.3 Cell Adhesion Molecules

The junction complexes present in all animal cells need protein structures that are highly specialized and controlled. These proteins belong to adhesion proteins and superfamilies. They have different features, such as ligands, anchoring functions, resistance, and cofactor functions. Hereafter, the major classes of proteins involved directly and indirectly in cell junctions will be classified according to their dependence on Ca^{2+}, and other proteins will be discussed during the explanation of junction complexes.

2.3.1 Ca^{2+}-Dependent CAMs

2.3.1.1 Selectins

Selectins are cell surface proteins which have affinity for carbohydrate (carbohydrate-binding proteins—lectins). They belong to the Ca^{2+}-dependent selection superfamily, the members of which are important for the formation of transient interactions between cells in blood vessels (Lii et al. 2010) (Fig. 2.4).

In vertebrates, important roles of selectins are inflammatory response and coordination of leukocytes (*leukocyte rolling*). Molecularly, selectins are transmembrane proteins with a greatly conserved selectin domain. Three types of selectins are found in abundance: L-selectin, P-selectin, and E-selectin (McEver 2003).

L-selectin is mostly found on leukocyte surfaces. In lymphocyte organs, endothelial cells express carbohydrates which bind to L-selectin, retaining these cells. In inflammatory response, endothelial vessel cells express L-selectin, which recognizes the saccharides of leukocytes, which is followed by leukocyte–vessel adhesion. The start of the diapedesis process is also assisted by specific integrin proteins. These interactions (of integrins and selectins) are heterophilic; selectins bind to specific oligosaccharides present on glycoproteins and glycolipids. However, the interaction between selectins and saccharides occurs with low affinity and, for this reason, is transient. Therefore, integrins are necessary.

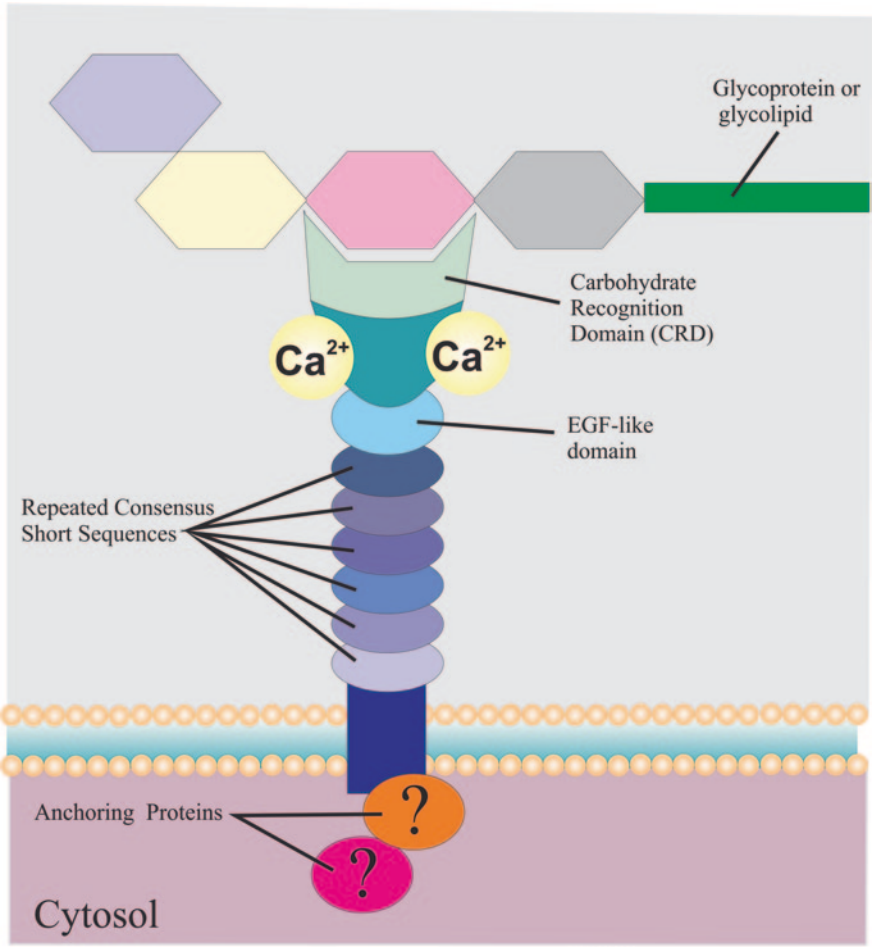

Fig. 2.4 Structure of selectins. Selectins, integrins, and intercellular adhesion molecules (ICAMs) perform an important role in inflammatory processes and migration of leukocytes (*homing*). They have three extracellular domains: the carbohydrate recognition domain, which is specific for saccharides presents in glycoproteins and glycolipids; the epidermal growth factor (*EGF*)-like domain, which is a repeated sequence region, a homologue of EGF; and repeated short consensus sequences. In the cell, there are yet unknown anchoring proteins which link selectin to the actin cytoskeleton

The major ligand for L-selectin is P-selectin glycoprotein ligand 1 (PSGL-1), a sialomucin expressed on leukocytes, and on glycoproteins found on high endothelial venules of Peyer's patches and of peripheral lymph nodes. L-selectin/PSGL-1 binding activates leukocyte aggregation, and binding to high endothelial venules initiates the transmigration necessary for lymphocyte homing. Also, P-selectin binds to PSGL-1, and this event is critical for linking and rolling of leukocytes on endothelium or surface-bound platelets (Sperandio et al. 2003).

During inflammatory response, P-selectin found on platelets and endothelial cells binds to the actin cytoskeleton through yet unknown anchoring proteins. Finally, E-selectin is expressed by active endothelial cells.

Selectins, like most lectins, bind to a series of glycoconjugates with different affinities. These glycoconjugates mediate cell adhesion to selectins under a physiological stream. All selectins bind with low affinity to glycans with terminal components that include α1,3-linked fucose and α2,3-linked sialic acid, and are characterized by the sialyl Lewis x (sLex) determinant (NeuAcα2,3Galβ1,3[Fucα1,3]GlcNAcβ1-R). Crystal structures of sLex bound to the lectin domains of P-selectin and E-selectin reveal a network of interactions between fucose, Ca^{2+}, and numerous amino acids, including those that coordinate Ca^{2+} binding, which explains the Ca^{2+} dependence for binding to fucosylated glycans. Galactose and sialic acid also bind to the lectin domain (Homeister et al. 2001).

2.3.1.2 Cadherins

Cadherins are present in several vertebrates and invertebrates, but not in some fungi, plants, and prokaryotes. They received the name "cadherin" because of their dependence on the presence of Ca^{2+}. If Ca^{2+} ions are removed from the medium, cadherins lose their Ca^{2+}-dependent adhesion function. The loss of adhesion in pathogenic conditions such as cancer is related to the invasive behavior of tumor cells.

The cadherins include a family of Ca^{2+}-dependent cell adhesion proteins that form and maintain adhesive contacts between the several types of cells (Fig. 2.5). They are, in general, single-pass transmembrane proteins containing different cadherin repeat motifs in their extracellular segments. The connections between cadherin domains are made by specific binding of Ca^{2+} ions between each domain pair. The presence of Ca^{2+} is necessary for adhesive function (Fig. 2.5).

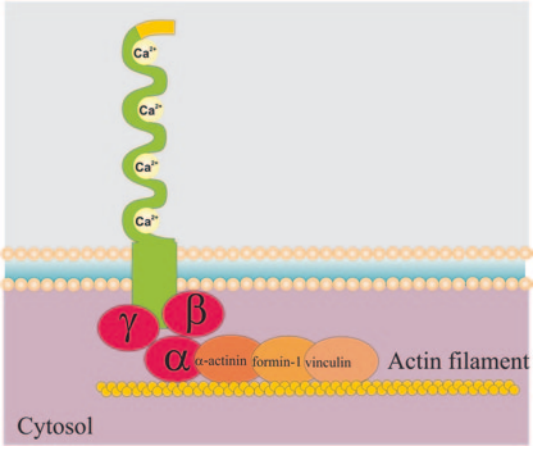

Fig. 2.5 Family of cadherins. Cadherins are the major adhesion proteins which keep cells joined in tissue. Extracellular domains bind to Ca^{2+} ions (cadherins are Ca^{2+}-dependent proteins). α-Catenin, β-catenin, and γ-catenin (in *red*) plus formin-1, vinculin, and α-actinin form the catenin complex. γ-Catenin can be replaced by p120 catenin

The extracellular domains form *trans* cadherin interactions between adjacent cells and initiate weak cell–cell adhesion and the formation of the junctions (Hartsock and Nelson 2008). Cadherins are classified into subfamilies based on the composition of the extracellular segments (see Table 2.1): the classical cadherins (or type I and type II cadherins), which are linked to the actin cytoskeleton; nonclassical cadherins, such as desmosomal cadherins (e.g., desmogleins and desmocollins), which are linked to intermediate filaments; and protocadherins, which are characterized by the presence of a variable number of extracellular domains, linked to a cytoplasmic tail which presents no homology with classical cadherins and does not bind to β-catenin or γ-catenin. A large subcategory of the protocadherins are encoded in new multigene loci under complex regulation (Patel et al. 2003).

Nonclassical cadherins with different sequences in the cadherin domain are more expressed in the brain (more than 50 types). These cadherins are also involved in adhesion; for example, protocadherins in the brain, and desmogleins and desmocollins in desmosomes. Some of these cadherins seem to have signaling function: T-cadherin, which is anchored by glycosylphosphatidylinositol without a transmembrane domain on the cellular membrane of nerve and muscle cells (Marthiens et al. 2002; Gomez et al. 2011).

The first three classical cadherins identified were named according to the tissue where they were found: E-cadherin, N-cadherin, and P-cadherin. E-cadherins in epithelial tissue are responsible for the maintenance of most of the epithelial layers. These proteins can be expressed in parts of the brain, and they exhibit a highly conserved cytoplasmic tail consisting of about 150 amino acids (Bremnes et al. 2002b).

This highly conserved cytoplasmic tail contains a binding site for either β-catenin and γ-catenin/plakoglobin, members of the superfamily of armadillo repeat proteins. E-cadherin and β-catenin form a complex by interaction between the cytoplasmic E-cadherin tail and 12 segments of β-catenin repeats of 42 amino acid armadillo sequences (Näthke et al. 1995). The N-terminal domain of β-catenin or γ-catenin directly binds to α-catenin. Since α-catenin is capable of binding to F-actin itself and to F-actin-binding proteins, such as α-actinin and vinculin, the E-cadherin–catenin complexes are consequently linked to the actin cytoskeleton (Bremnes et al. 2002b).

N-cadherins are mostly found in nerve, muscle, and connective tissues, but are also present in crystalline tissue. Nonclassical cadherins, protocadherins, can be found on brain cells (Vania 2002). The cadherin function seems to be important from the embryonic period as it allows adhesion of the embryonic cells in the first stages of development. A type of ubiquitous cadherin is VE-cadherin in endothelial cells, and a specific cadherin, P-cadherin, is found in placenta and epidermal tissue (Aplin et al. 2009).

The function of cadherins in cell adhesion occurs by a mechanism focused on β-strand exchange between monomers and the insertion of hydrophobic residues into the core of the partner molecule. This mechanism of interaction is common to cadherins known to function in cell adhesion, and specificity has probably arisen through changes in residues peripheral to these central interface elements. The human genome contains about 100 cadherin genes and it seems possible that those with intercellular adhesion functions operate through this common mechanism (Patel et al. 2003).

Table 2.1 Cadherin superfamily

Type	Location	Junction	References
Classical cadherins			
E-cadherin	Epithelia	Adherens junctions	Fahraeus et al. (1992), Pignatelli et al. (1994), Berx and van Roy (2001), Bremnes et al. (2002b), Hulpiau and van Roy (2009)
N-cadherin	Neurons, cardiac and skeletal muscle, and fibroblasts	Adherens junctions and chemistry synapses	Bremnes et al. (2002a), Hulpiau and van Roy (2009)
P-cadherin	Placenta, mammary epithelium, and epidermis	Adherens junctions	Paredes et al. (2007), Samuelov et al. (2012)
VE-cadherin	Endothelial cells	Adherens junctions	Herwig et al. (2008), Etienne-Manneville (2011)
Nonclassical cadherins			
Desmocollin	Skin	Desmosomes	Hulpiau and van Roy (2009), Feher (2012)
Desmoglein	Skin	Desmosomes	Hulpiau and van Roy (2009), Feher (2012)
T-cadherin	Neurons, cardiac muscle, and skeletal muscle	–	Ivanov et al. (2004)
Protocadherins α, β, and γ	Neurons	Synapses and others membranes	Emond et al. (2009), Hulpiau and van Roy (2009)
Flamingo	Sensorial epithelium and others	Cell–cell junctions	Usui et al. (1999), Shimada et al. (2001)
23 cadherin	Some epitheliums and inner ear	Sensorial pilus cells and stereocilium cells	Lagziel et al. (2005), Goodyear et al. (2010), Alagramam et al. (2011)
Fat	CNS cells and other tissues	Renal glomerulus and other cell junctions	Magg et al. (2005), Hulpiau and van Roy (2009)

Cadherin-specific intracellular partners such as kinases or phosphatases may interact with the cadherin–catenin complex and regulate its activity and intracellular signaling. It is also likely that cadherin function variations in different cell types depend on the cadherin repertoire of the cell. Moreover, cadherins can associate with growth factor receptors and modulate their signaling transductions. For example, VE-cadherin associates with vascular endothelial growth factor receptor 2 and controls its signaling pathways. Certainly, in the absence of VE-cadherin, vascular endothelial growth factor receptor 2 stimulates growth of endothelium in a deregulated form, altering the vascular development (Francavilla et al. 2009).

Homophilic Adhesion of Cadherins

A homophilic ligation means that a molecule binds or interacts with other molecules of the same type. In the case of cadherins, these proteins interact with other cadherin molecules of an adjacent cell, both cadherins being of the same subtype. The space between cellular membranes is defined precisely by the structure of partner cadherins (Fig. 2.6) (Hulpiau and van Roy 2009).

The ligation occurs in N-terminal regions of cadherins, in regions more distant from cell superficies. The space between membranes depends on the type of cadherin, mainly in anchoring junctions. All cadherins have a domain named the"cadherin domain," which is a region in the extracellular portion, typical for vertebrates (with five repeats), and also present in desmogleins and desmocollins (four or five repeats); however, more than 30 repeats in nonclassical cadherins can be found (Näthke et al. 1995).

Each domain forms a less rigid segment, linked to the next mobile domain, sites where Ca^{2+} ions are bound, blocking the movement and forming a ligation, a little curved but rigid.

Catenin–Cadherin Linkage to the Cytoskeleton

Although extracellular domains of cadherins are responsible for hemophilic interactions, intracellular domains in classical and nonclassical cadherins perform the anchorage to the cytoskeleton by actin filaments. However, these ligations are not direct; they occur through intracellular anchoring proteins. This group varies according to the anchoring junctions, but a key function has been attributed to α-catenin, β-catenin, and γ-catenin (plakoglobin). The p120 catenin helps with the contact between these catenins and cadherins (Näthke et al. 1995). β-Catenin is bound principally to desmosomes, and it is also critically involved in Wnt signaling. A collapse in the junction and consequent fragmentation can release β-catenin, which acts as a signaling molecule (by degradation or phosphorylation in theWnt signaling pathway) (Vania 2002).

α-Catenin regulates the association of proteins that connect E-cadherin and β-catenin complexes with the actin filaments. α-Catenin itself can associate with F-actin by direct binding through its C-terminal domain, but it can also associate directly with afadin, α-actinin, vinculin, and the zyxin family members (Etienne-Manneville 2011).

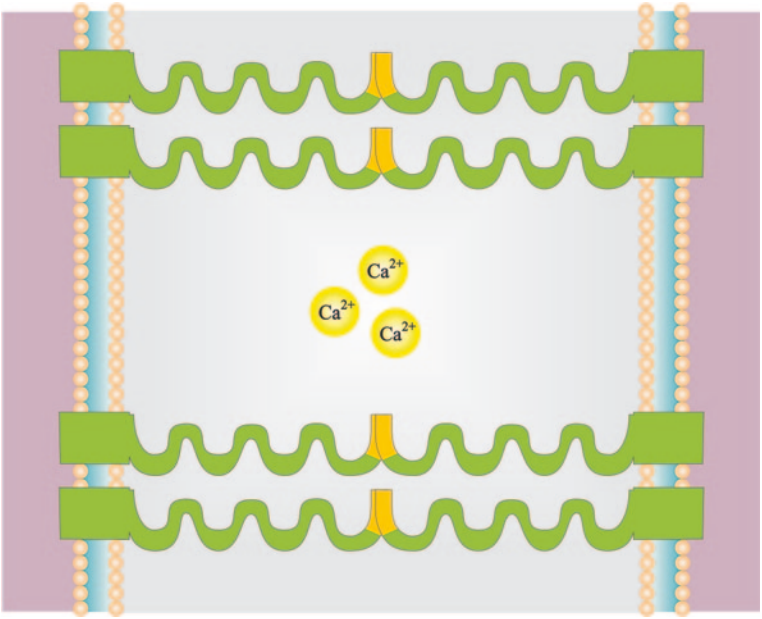

Fig. 2.6 Homophilic interactions between cadherins. At the binding face of cadherin, there is a sequence (in *yellow*) which allows the formation of homophilic *trans* dimers between cells, and homophilic *cis* dimers between partner cadherins in a single cell

The binding of α-catenin to afadin can attract adhesion complexes built by E-cadherin and nectins to the adhesion junctions. The binding to α-actinin, vinculin, and the zyxin family members can reinforce the association of E-cadherin-based adhesion junctions with F-actin, and can also employ the Ena/Vasp families of profilin–actin-binding proteins (Mandai et al. 1997). These machineries are vital for stabilizing cell–cell adhesion and organizing actin dynamics at these sites.

γ-Catenin contacts specially with desmosomal cadherins, like β-catenin; they can be replaced between themselves. p120 catenin was originally classified as a Src-tyrosine receptor kinase and a member of the catenin family on the basis of sequence homology to an armadillo domain of β-catenin. Binding of the p120 Arm domain to E-cadherin appears to be essential to conserve adequate cadherin levels for strong adhesion (Etienne-Manneville 2011). It also functions as a regulator of cell motility through the actin cytoskeleton by interacting with Rho family GTPases.

2.3.1.3 Integrins

Integrins play many roles during cell development. All nucleated cells in the body have a precise collection of integrins; the roles of these proteins are complex and are integrated with the specific functions of the cells in each tissue. The functions of integrins at the cellular level can be categorized into those related to their

Table 2.2 Mammalian integrin heterodimers

Integrin	Location	Principal ligand
$\alpha_3\beta_1$	Skin, kidney, lung, cortex	Laminin
$\alpha_6\beta_1$	Gametes, macrophages, platelets	Laminin, ADAMs, hemidesmosomes, epithelia
$\alpha_7\beta_1$	Muscle	Laminin
$\alpha_5\beta_1$	Blood vessels (during embryogenesis)	Fibronectin
$\alpha_8\beta_1$	Kidney, inner ear	Fibronectin, vitronectin, tenascin C
$\alpha_V\beta_1$	Diffuse, not clear	Fibronectin, vitronectin
$\alpha_V\beta_3$	Osteoclasts	Fibrinogen, fibronectin, vitronectin, tenascin C, osteopontin, bone sialoprotein, MMP-2
$\alpha_V\beta_5$	Retina, bones	Vitronectin
$\alpha_V\beta_6$	Skin, lungs	Fibronectin, TGF-β–LAP
$\alpha_V\beta_8$	Vessels (during development)	Vitronectin
$\alpha_{IIb}\beta_3$	Platelets	Fibrinogen, fibronectin, vitronectin
$\alpha_4\beta_1$	Heart (during development)	Fibronectin, VCAM
$\alpha_4\beta_7$	Peyer's patches	Fibronectin, VCAM, MadCaM
$\alpha_9\beta_1$	Lymph nodes (during development)	Tenascin C, osteopontin, ADAMs, factor XIII, VCAM, VEGF-C, VEGF-D
$\alpha_1\beta_1$	Mesenchymal cells	Collagens, semaphorin 7A,
$\alpha_2\beta_1$	Platelets, epithelium, mesenchymal cells	Collagens, tenascin C,
$\alpha_{10}\beta_1$	Cartilaginous tissue	Collagens
$\alpha_{11}\beta_1$	Collagens	Periodontal ligaments
$\alpha_D\beta_2$	Eosinophils	ICAM, VCAM
$\alpha_M\beta_2$	Leukocytes	ICAM, VCAM, iC3b, factor X, fibrinogen
$\alpha_L\beta_2$	Leukocytes	ICAMs
$\alpha_X\beta_2$	Leukocytes	Fibrinogen, plasminogen, heparin, iC3b
$\alpha_E\beta_7$	Skin, gut	E-cadherin

ADAM a disintegrin and metalloprotease, *MMP-2* matrix metalloprotease 2, *TGF-β* transforming growth factor β, *LAP* latency-associated peptide, *VCAM* vascular cell adhesion molecule, *ICAM* intercellular adhesion molecule, *MadCAM* mucosal addressin cell adhesion molecule, *VEGF* vascular endothelial growth factor

roles as mechanical regulators in cell adhesion and migration and their signaling function (Bokel and Brown 2002; Hynes 2002). The integrin-dependent effect on signaling pathways in some cell types influences cytoskeletal mobility, expression activity, and a variety of alterations in metabolism (Bokel and Brown 2002).

Integrins are heterodimers composed of α and β subunits. Mammals are known to have 24 types: 18 different integrin α subunits and eight β subunits. The model of integrin synthesis indicates that the promiscuous subunits are produced in a large excess when associated with the other subunits (Hynes 2002), forming different combinations of integrin subunits (Table 2.2). In the endoplasmic reticulum, integrin subunits find their binding partners and form heterodimers.

The general functions of integrins are cell adhesion and communication. In vertebrates, integrin subunits have differentiated to accommodate additional functions with roles that are likely related to the large changes that occurred during chordate evolution (Brown 2000; Johnson et al. 2009). Integrins bind ECM proteins through their large external domain and engage the cytoskeleton via their cytoplasmic terminals.

These integrin-mediated connections on both sides of the membrane are dynamically linked; the cytoskeleton controls the affinity of the integrin external domain and thus modulates the ECM, and integrin binding to the ECM changes the form and composition of the cytoskeleton. The affinity of integrin ligands is directly related to the presence of bivalent ions such as Ca^{2+} and Mg^{2+} (Fig. 2.7). Different concentrations of these ions increase or decrease the affinity of integrins for their ligands. Forces generated as a result of cytoskeleton contraction or ECM rigidity are applied across the plasma membrane through integrins and contribute to the conformational changes of these receptors and their anchoring proteins, and to the nature of signal transmission (Johnson et al. 2009; Janik et al. 2010).

Except for one type of integrin, all types of human integrins are linked to actin filaments, through talin and a set of other intracellular anchorage proteins. The actin-linked cell–matrix junctions formed by integrins may be small and temporary, or large and strong. Binding of ligands is associated with changes of conformation.

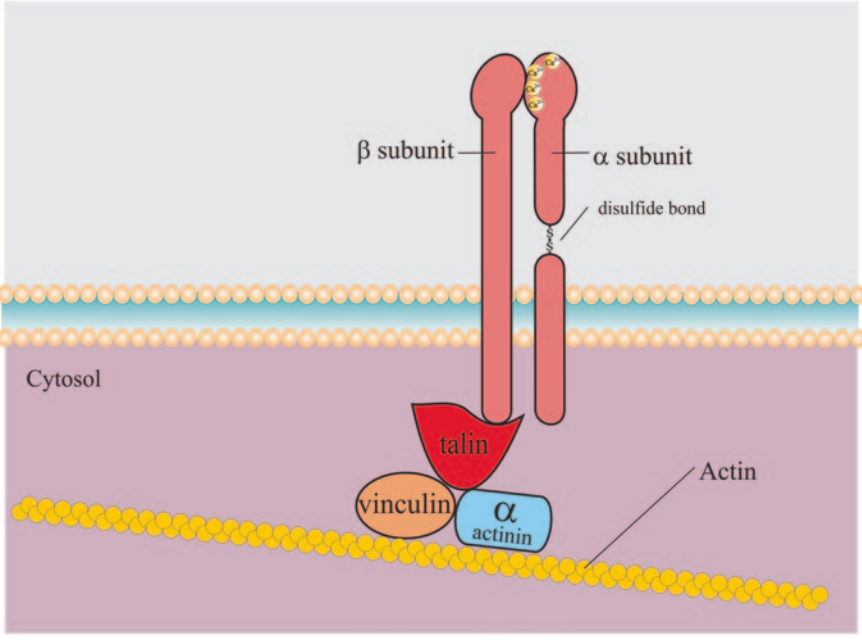

Fig. 2.7 Structure of integrins. Integrins have two functions: they bind to the ECM and to the cytoskeleton. Molecularly, they have two subunits; the α subunit, with two chains linked through a disulfide bond and multiple sites for ligation of Ca^{2+} (divalent-ion-dependent protein), and the β subunit, which has an intracellular region that interacts with actin filaments through talin, vinculin, and α-actinin or filamin

This generates an allosteric coupling between binding the ECM to the cell and binding it to the cytoskeleton, allowing the integrin to transport signals in both directions across the plasma membrane. Binding of the intracellular anchor protein talin to the terminal of an integrin tends to drive the integrin into an extended conformation with increased affinity for its extracellular ligand (Liu et al. 2000; Arnaout et al. 2007).

Equally, binding to an extracellular ligand, by promoting the same conformational modification, leads to binding of talin and formation of a linkage to the actin cytoskeleton. Complex associations of proteins become organized around the intracellular terminal of integrins, producing intracellular signals that can influence almost any aspect of cell behavior, including proliferation and survival as well as the phenomenon of anchoring dependence of cell polarity and guidance of migration.

The backward pull of actin filaments by myosin motors applies force on the initial ECM–integrin–cytoskeleton linkages, causing fast accumulation of anchoring proteins that transform focal complexes into cell adhesions, with larger contact developing on firmer matrices, thus directing cell movement. The ligations of integrins are modified constantly, a normal phenomenon in the ECM which ensures the remodeling of the ECM. Reversion of these ligations is caused by a disintegrin and metalloprotease (ADAM)) with important roles during angiogenesis, fertilization, neurogenesis, cardiogenesis and tumorigenesis. ADAM has an intracellular and an extracellular domain, exhibiting regions with disintegrin and/or metalloprotease function. Disintegrin regions compete with integrins for ligation with laminin, fibronectin, and other ECM proteins, whereas metalloprotease regions are responsible for ECM degradation and migration (Johnson et al. 2009).

2.3.2 Ca^{2+}-Independent CAMs: Immunoglobulin Superfamily

Proteins of the immunoglobulin superfamily (immunoglobulin-like CAMs) are composed of Ca^{2+}-independent surface proteins which form connections between cells (cell–cell adhesion) by heterophilic and homophilic interactions. These proteins have immunoglobulin-like domains with several functions, different from immune responses (Lii et al. 2010). They are defined by the presence of one or more copies of the immunoglobulin motif, a compact structure with two cysteine residues separated by 55–75 amino acids arranged as two antiparallel β sheets (Francavilla et al. 2009). Some immunoglobulin-like CAMs also have one or more copies of a fibronectin type III repeat (e.g., neural CAMs, NCAMs, which are described below).

Immunoglobulin-like CAMs typically have a large N-terminal extracellular domain, a single transmembrane helical segment, and a cytoplasmic tail. Intercellular adhesion molecules and vascular CAMs are the main CAMs recognized by leukocyte integrins by heterophilic binding, whereas NCAMs (Fig. 2.8), which are expressed in several types of tissue and in most neural cells and are

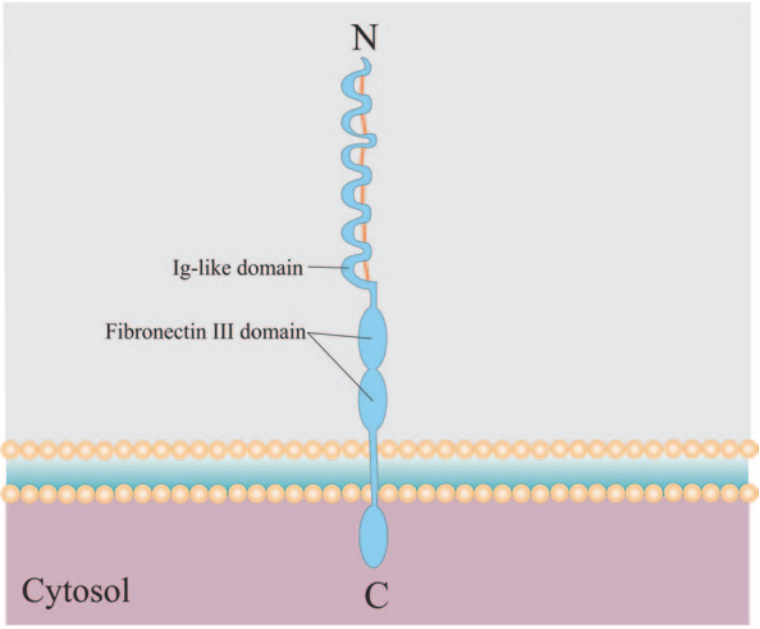

Fig. 2.8 Immunoglobulin (*Ig*) superfamily. Neural CAM, an example of an Ig-like CAM. This protein is expressed in neurons and other cell types. It has sialic acid covalently bound to lateral chains, avoiding adhesion

produced in different versions because of alternative splicing, form homophilic interactions. NCAM and L1, which is responsible for nervous system development, are specifically involved in axon guidance and in the establishment and maintenance of synapses (Francavilla et al. 2009).

Interactions exerted by CAMs are not as strong as integrin binding is, but it seems that CAMs contribute to regulation of adhesion, principally during regeneration and growth.

2.4 Anchoring Junctions

Two groups of adhesion molecules are involved in cell anchoring: Ca^{2+}-dependent molecules, which include cadherins and selectins, and Ca^{2+}-independent molecules, represented by the integrin and immunoglobulin superfamily.

Selectins, in particular, are involved in saccharide recognition (belonging to the lectin group), besides being involved in movement of leukocytes into blood vessels—diapedesis—when there is inflammation. There are three types of selectins on the cell surface: P-selectin, which is stored in platelets and is found in active endothelial cells; E-selectin, which is found in active endothelial cells; and L-selectin, which is found in leukocytes.

Cells use several types of adhesion molecules for cell–cell adhesion and cell–ECM adhesion. Integrins are involved, primarily, in cell–ECM interactions, whereas cadherins establish an interaction with the inner cytoskeletonand the exterior surface.

2.4.1 Adherens Junctions

The adherens junctions are responsible for multiple functions such as initiation and maintenance of cell–cell adhesions, regulation of the cytoskeleton, signaling transduction, and expression regulation. There are interactions among the classical cadherin superfamily, such as E-cadherin, and the catenin family members including p120 catenin, α-catenin, and β-catenin (Fig. 2.9). These proteins control the formation, preservation, and function of adherens junctions (Hartsock and Nelson 2008).

Intercellular junctions develop at the cell-to-cell contact sites between adjacent cells, and several types of junctions, including tight junctions, adherens junctions, and desmosomes, occur in epithelial cells. These junctions form junctional complexes at the most apical part of the lateral membrane (Nagafuchi 2001).

Adherens junctions are cell–cell adhesion sites where classical cadherins function and where the actin cytoskeleton and some cytoplasmic components are assembled (Niessen and Gottardi 2008). The cadherin is directly bound to β-catenin or γ-catenin, which in turn binds to α-catenin; α-catenin then binds to vinculin, α-actinin, zonula occludens (ZO)-1, and actin filaments (Hartsock and Nelson 2008). Additionally, nectin, and its associated l-afadin are also found concentrated at adherens junctions. Multimolecular arrangements composed of these molecules are thought to serve as a structure for adherens junctions (Mandai et al. 1997).

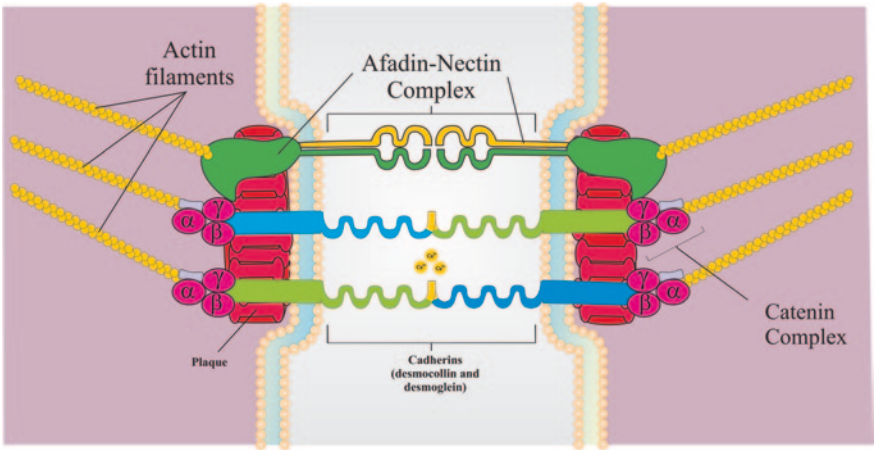

Fig. 2.9 Zonula adherens. The zonula adherens is analogous to a sash (or cincture); hence, it is also called the desmosome sash. This junction is associated with actin filaments through cadherins (desmocollins and desmogleins) and with the catenin complex (α-catenin, β-catenin, and γ-catenin)

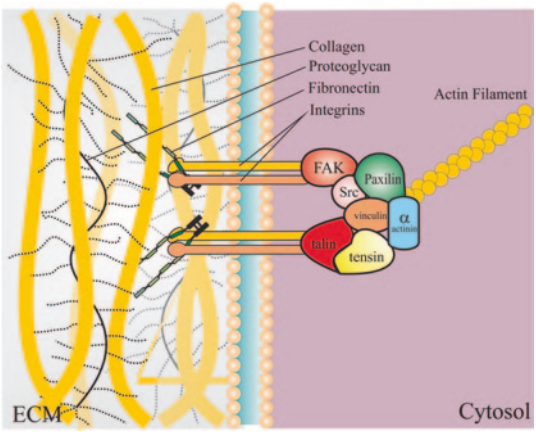

Fig. 2.10 Focal adhesion structure. Focal adhesions have at least two significant cellular functions: to transmit tension at adhesion locations to maintain strong attachments to the ECM, and to be signaling centers from which signaling intracellular pathways regulate cell growth, survival, and gene expression. *FAK* focal adhesion kinase

2.4.2 Focal Adhesions

Whereas adherens junctions are transposable from cell to cell, focal adhesionsare responsible for cell–ECM interactions. Molecularly, these structures are very similar, with some peculiarities. Focal adhesions are specialized sites where integrin receptors link the ECM to the actin cytoskeleton. Focal adhesions have at least two significant cellular functions: to transmit force or tension at adhesion sites, maintaining strong attachments to the underlying ECM, and to act as signaling centers from which numerous intracellular pathways emanate to regulate cell growth, survival, and gene expression (Sastry and Burridge 2000; Zhao and Guan 2011).

Integrins cluster into complexes with structural, cytoskeletal proteins such as talin, vinculin, and α-actinin, besides signaling molecules such as p130cas, focal adhesion kinase (FAK), and paxillin (Fig. 2.10).

Focal adhesions are structures that assemble, disperse, and recycle as cells migrate or enter mitosis. Recent evidence reveals the complexity of these processes. The degree of assembly is related to the regulation of Rho family GTPases through cross talk between integrins and several adhesion receptors such as syndecans, G-protein-coupled receptors, and receptor tyrosine kinases, as well as the interaction between microtubules and actin filaments. It also seems that focal adhesions are themselves motile and heterogeneous in composition. Lastly, turnover of focal adhesions involves communication with mechanisms of vesicle traffic pathways and microtubules (Sastry and Burridge 2000).

2.4.3 Desmosomes

The ECM is not just an ultrastructure that is flexible and moldable for the requirements of the cell, it also maintains its influence on cells in biological processes such

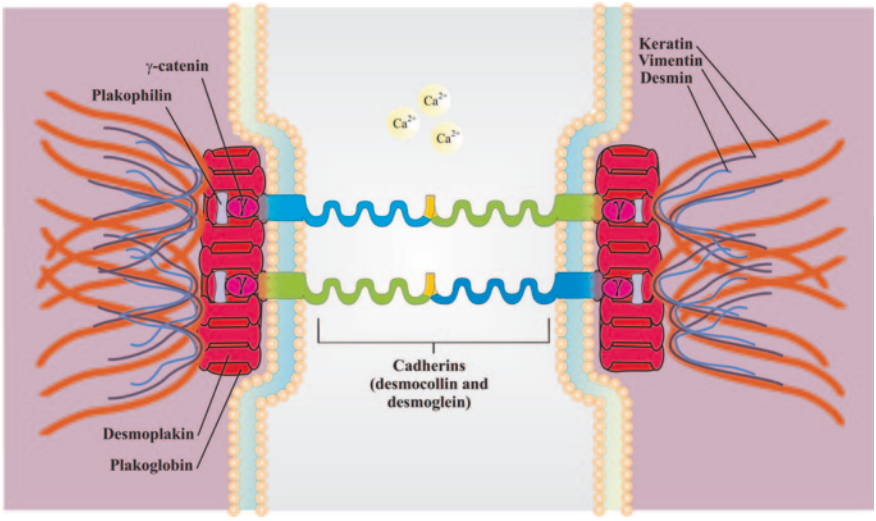

Fig. 2.11 Structure of the desmosome. γ-Catenin interacts directly with a region of nonclassical cadherins (desmocollin and desmoglein), desmoplakin, and plakophilins. Plakophilins recruit proteins to the plasma membrane. Desmoplakins form connections between cadherins (intracellular segments) and intermediate filaments such as keratin, vimentin, and desmin

as cell adhesion, migration, proliferation, differentiation, and survival, modulating the bioactivities of growth factors and cytokines, sequestering growth factors, or directly affecting receptor activities (Martino et al. 2012). Binding between transmembrane adhesion proteins and the cytoskeleton provides stability for the cell.

The nature of desmosomes (or adhesion spots) is very similar to adhesion by cadherins, but they use intermediate filaments and not actin filaments. Desmosomes guarantee mechanical strength for the cell, mainly in vertebrates, given by the connection of an intermediate filament net between adjacent cells (Fig. 2.11).

At the cytoplasmic surface of each cell connection, there is a plaque composed of intracellular anchoring proteins. A bundle of intermediate filaments is bound to the surface of each plaque. Adhesion transmembrane proteins such as cadherins link to plaques in the presence of Ca^{2+} ions. The intermediate filaments which participate in the linkage to desmosomes depend on the cell type, such as keratin filaments in epithelial layers or desmin filaments in cardiac muscle.

Desmogleins and desmocollins are the major molecular components of desmosomes. They are cadherin proteins, and their cytoplasmic tails bind to γ-catenin and plakophilin, or even to desmoplakin. Desmoplakin binds laterally to intermediate filaments, attaching desmosomes to these filaments (Vania 2002).

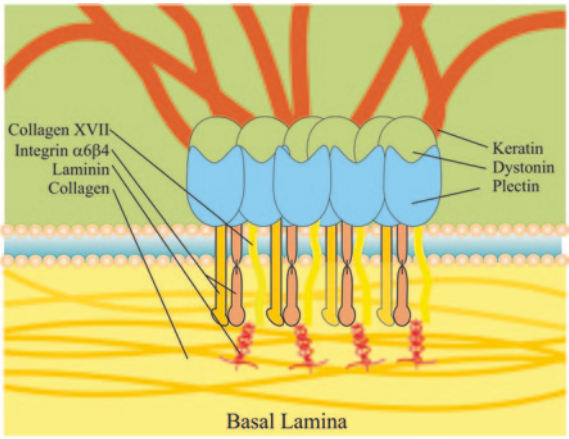

Fig. 2.12 Structure of hemidesmosomes. Epithelial cells are immobilized to the basal lamina by hemidesmosomes, binding keratin filaments to laminin. An integrin $\alpha_6\beta_4$ crosses the membrane, binding to intracellular keratin filaments through plectin and dystonin to extracellular laminin. Together with integrin $\alpha_6\beta_4$, there is collagen type XVII

2.4.4 Hemidesmosomes

Cells are linked to the basal lamina by hemidesmosomes. These structures bind the keratin cytoskeleton (a kind of intermediate filament) at the inner side of the cell to laminin on the external side of the cell (Fig. 2.12).

Hemidesmosomes are composed of a highly specialized transmembrane $\alpha_6\beta_4$ integrin, which links keratin filaments of the cytoskeleton by plectin and dystonin to external laminin 5. Collagen type XVII can also be found in an adhesive complex; it has a transmembrane domain that binds to collagen at the ECM.

2.5 Occluding Junctions (Tight Junctions)

Cell polarity is fundamental for cell functions, such as ion and nutrient transport, secretion of products, and protection. Parts of these functions are performed by occluding junctions or tight junctions. These junctions are localized at the apical end of the basolateral membrane, and play key roles to create the cell polarity (Chiba et al. 2008).

Tight junctions form barriers to prevent free passage of molecules between cells through canals between them, and also act as boundaries between apical and basolateral membranes, preventing diffusion of components between the apical and basolateral domains. These sealing strands correspond to fusion sites of opposing plasma membranes and are composed of transmembrane adhesion proteins. Adhesion junctions form continuous adhesion belts localized near the apical end of the cell, just below tight junctions. Three distinct classes of transmembrane

proteins have been localized at tight junctions: occludins, claudins and junctional adhesion molecules (JAMs), and will be described next (Miyoshi and Takai 2005).

In tight junctions, there are also tricellulin proteins, which are the latest integral protein identified at the tight junction. They are concentrated at the vertically oriented tight junction strands of tricellular contacts and seem to be essential to seal the paracellular membrane (González-Mariscal et al. 2007).

2.5.1 Occludins, Claudins, and JAMs

Occludin has four transmembrane domains and two extracellular loops, with the N- and C-termini facing the cytoplasm. The role of occludin in the barrier or fence functions of tight junctions appears to be inconclusive.

Tight junctions are thought to be membrane microdomains or microdomain rafts. The raft is a dynamic assembly of cholesterol and sphingolipids in the plasma membrane that plays an important function in the organization of these junctions. Since hyperphosphorylated occludin has been identified in major pools of microdomains and forms a complex with ZO-1, occludin perhaps takes part in the regulation of paracellular permeability in epithelial cells (Chiba et al. 2008; Kirschner et al. 2010). Besides ZO-1, occludins interacts with ZO-2, ZO-3, and afadin (Fig. 2.13).

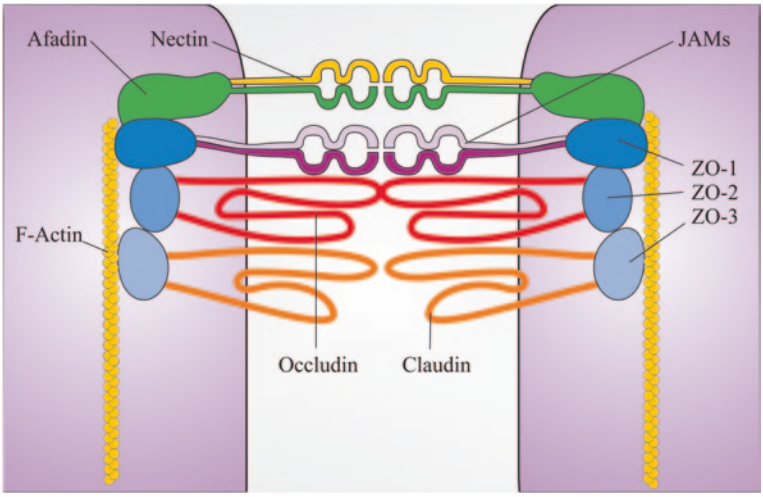

Fig. 2.13 Components of a zonula occludens. Tight junctions seal spaces between cells and regulate the passage of ions. Occludins and claudins are the molecular basis for the formation of tight junctions. These proteins have four transmembrane domains. Nectin and junctional adhesion molecules (*JAMs*) help intercellular adhesion: JAMs are associated with afadin and zonula occludens 1 (*ZO-1*), determinating the cell polarity; nectin forms the nectin–afadin complex, anchored on ZO-1. *ZO-2* zonula occludens 2, *ZO-3* zonula occludens 3

Claudins (Latin *claudere*, meaning "to lock," "to seal") have been identified as the central structural and functional components of tight junctions involved in paracellular transport. They are localized at the site of near membrane apposition within tight junctions. These proteins are detected in both epithelial and endothelial cells in all tissues that tolerate tight junctions, and form a complex with occludin and JAMs.

Claudins are small proteins of 24 kDa (maximum size), with two extracellular domains, four transmembrane domains, and cytoplasmic N- and C-termini (Rizzolo et al. 2011). The C-terminus of most claudins ends in a supposed PDZ-binding domain, and claudins interact directly with the first PDZ domains of ZO-1, ZO-2, and ZO-3 (Kirschner et al. 2010). Claudins interact indirectly with the myosin-binding molecule cingulin. Claudins generate a series of regulated channels within tight junction membranes for the passage of ions and small molecules. Moreover, claudin defects have been implicated in a wide range of human cancers.

Finally, JAMs are immunoglobulin-like single-span transmembrane molecules and mediate Ca^{2+}-independent adhesion. They are concentrated at tight junctions as well as at adhesion junctions (Näthke et al. 1995). JAMs form linear groups at cell–cell adhesion positions, but do not form strand-like structures as observed with claudins (Rizzolo et al. 2011). JAM-1 is localized with F-actin at the cell–cell interactions and at the membrane ruffles. It facilitates cell adhesion through homophilic binding, and regulates junction permeability and lymphocyte trafficking as a ligand for β_2 integrin lymphocyte-function-associated antigen 1 (LFA-1). JAM-2 is a vascular molecule expressed by endothelial and lymphatic cells. JAM-2 is localized to endothelial venules within lymph nodes and Peyer's patches of adult mice, and plays a central role in regulation of diapedesis. JAM-3 is expressed in human platelets and is known as a counter-receptor for the leukocyte β_2 integrin Mac-1 intermediating leukocyte–platelet interactions. JAM-4 is localized to tight junctions and apical membranes of epithelial cells in the jejunum, ileum, and renal proximal tubules. JAM-4 interacts with membrane-associated guanylate kinase interacting protein like 1 (MAGI-1), a scaffolding protein at tight junctions, which may regulate the permeability of kidney glomerulus and small-intestinal epithelial cells (Kirschner et al. 2010).

The extracellular domains of JAMs consist of two V-type immunoglobulin domains. JAM-1 forms homophilic *cis* dimers and homophilic *trans* dimers, which contribute to adhesive function and junctional organization at tight junctions. JAM-2 and JAM-3 are heterophilic binding partners, although they do not undergo homophilic interactions. JAM-2 adheres to T cells through heterotypic interactions with JAM-3. JAMs might be novel molecular targets for antagonizing interactions between hematopoietic and endothelial cells that promote inflammatory vascular diseases (Kirschner et al. 2010; Rizzolo et al. 2011).

Claudins and JAMs have a valine residue at their cytoplasmic C-terminal portion and bind ZO proteins through their PDZ domains, whereas occludin binds them through their guanylate kinase domain (González-Mariscal et al. 2007). Since ZO-1 and ZO-2 are F-actin-binding proteins and form a heterophilic dimer with ZO-3, occludin, claudins, and JAMs are all linked to the actin cytoskeleton through these adaptor proteins. Tight junctions also concentrate signaling proteins

and cell polarity proteins, and serve as a monitoring center to help in coordinating multiple cell processes (Chiba et al. 2008).

ZO proteins, junction enriched and associated protein (JEAP), and some other proteins act as adaptors at the cytoplasmic surface of tight junctions. The ZO proteins bind the tight junction transmembrane proteins and F-actin with their N- and C-termini, respectively. This complex appears to link tight junctions to an actomyosin ring, which supports and regulates tight junction permeability in epithelial cells. ZO-1 links E-cadherin to the actin cytoskeleton indirectly through α-catenin, which is known as one of many F-actin-binding proteins, or interacts with connexins, which are the transmembrane molecules of gap junctions, depending on the cell type. ZO-1 is a 220-kDa phosphoprotein that contains three PDZ domains, a Src homology 3 domain, and an inactive guanylate kinase domain. Multidomain ZO proteins can bind numerous different protein partners to control the dynamics of tight junction assembly, which requires the clustering of occludin and claudins.

JEAP is a newly identified component of tight junctions that is specifically expressed in exocrine cells. JEAP contains a polyglutamic acid repeat at the N-terminal region, a coiled-coil domain in the central region, and a consensus motif for binding to PDZ domains in the C-terminal region (Miyoshi and Takai 2005). Although the exact functions of the components of tight junctions are unknown at present , the presence of multiple PDZ domains in some tight junction proteins could elucidate the complexity of protein–protein associations.

2.6 Communicating Junctions

The communicating junctions or gap junctions are found ubiquitously on connective and epithelial tissues and are responsible for cell–cell communications by small signaling molecules. The trafficking of these molecules is possible because the presence of a central chaperone consisting of connexin and innexin protein family members (there are 21 types of these proteins in humans).

Gap junctions provide a passage for direct intercellular communication. The flow of molecules through these channels is called gap junctional intercellular communication and includes passive diffusion of small (less than 1 kDa) and hydrophilic molecules, such as metabolites (ATP), nutrients (e.g., glucose), and second messengers (e.g., inositol 1,4,5-trisphosphate and Ca^{2+}). (Alexander and Goldberg 2003; Vinken et al. 2006). As numerous physiological processes are mediated by regulatory molecules that are exchanged via gap junctions, gap junctional intercellular communication is considered a key instrument in the control of all aspects of cellular life (Vinken et al. 2006).

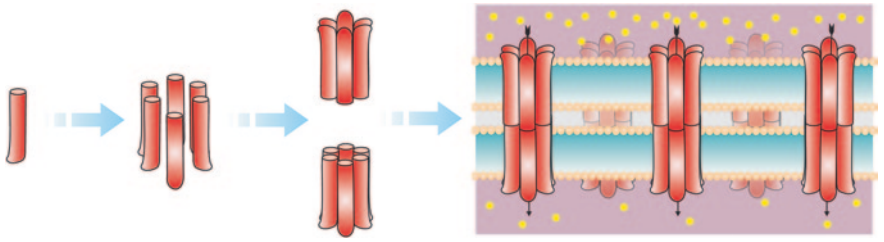

Fig. 2.14 Gap junctions. Six connexin monomers organize themselves to form a hexameric, cylindrical connexon, with a central channel. These connexons align with other connexons of an adjacent cell, forming an intercellular channel between the cytoplasms. The intercellular channel allows passage of small molecules which coordinate cellular responses. Agglomerated channels form the communicating junctions or gap junctions

2.6.1 Connexins to Connexons

Gap junctions arise from the interaction of two opposing connexons, in turn consisting of six connexin proteins. Twenty types of connexins can be found in humans. They all share an analogous structure of four membrane-spanning domains, two extracellular loops, one cytoplasmic loop, one cytosolic N-terminal tail, and one C-terminal region (Fig. 2.14). Variety between the connexin family members is mainly due to structural differences within the cytosolic areas (Hua et al. 2003; Batra et al. 2011).

Connexins are normally named according to their size, based on complementray DNA sequence information. The most abundant hepatic connexin has a predicted molecular mass of 32 kDa and is therefore called Cx32. Several connexin isoforms seem to be based on specific signals located within the connexin polypeptides. Binding between two connexons occurs via noncovalent interactions between the extracellular segments of apposing connexins, which contain three conserved cysteine residues, with each of them forming intramolecular bridges (Herve et al. 2007; Kovacs et al. 2007).

The two connexons composing the junctional channel are rotationally staggered with respect to each other so that the α helices of each connexin are axially aligned with the α helices of two nearby monomers in the apposed connexon. The possibility of forming heteromeric channels by connexons composed of different connexins was found predominantly for Cx40, Cx43 and Cx45 did not form such channels, whereas some others were able to associate into functional channels.

Connexons and connexins interact with specific cytoplasmic proteins that link them to the cytoskeleton and to intracellular signal transduction pathways. Association with intracellular molecules is likely to be important for immobilization and clustering of the channels between cells, for correct directing of channels to specific cellular locations, for enabling channels to shaft ions and small molecules, and for modulation of the channel functions by kinases, phosphatases, and other regulatory proteins, in addition to other unknown functions.

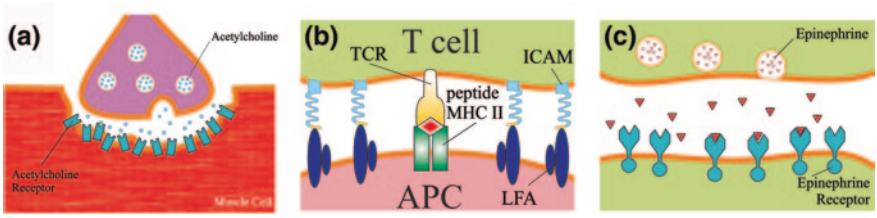

Fig. 2.15 Signaling junctions. *APC* antigen-presenting cell, *LFA* lymphocyte-function-associated antigen, *TCR* T-cell receptor

2.7 Signaling Junctions

Signaling junctions are responsible for signal transmission through the plasma membrane between cells on contact spots. Anchoring proteins and proteins capable of mediating signal transmission participate. An example of this type of junction is the neuromuscular junction.

In this chapter, signaling junctions will be divided into three groups (Fig. 2.15): chemical synapses (in the nervous system), immunological synapses (in the immune system) and cell–cell signaling contact (e.g., Delta–Notch) (Werle 2008; Wu et al. 2010).

2.7.1 Chemical Synapses

Chemical synapses are present in large numbers in the nervous system. The brain contains billions of neurons and the contacts between these cells and a target cell are built by synapses. These asymmetric structures are the place where neurotransmitters are released from the presynaptic cell and activate receptors on the postsynaptic cell, establishing communication between cells. Here, the neuromuscular junction (synapse between motor neurons and skeletal muscle fibers) will be the example explored.

The motor neuron has a region called a "active zone" on its membrane, where ´neurotransmitter vesicle fusion occurs, The type of neurotransmitter released differs among species (in *Drosophila* glutamate, and in vertebrates acetylcholine) (Wu et al. 2010).

The ECM is important in this type of chemical synapse signaling junction not only as a scaffold that maintains the spatial interaction between the motor neuron and the muscle cell; it also participates in cell-to-cell signaling. Agrin (a heparan sulfate proteoglycan produced by motor neurons) belongs to the synapse matrix and plays a role in aggregation of acetylcholine receptors in the muscle cell membrane interactivity with muscle-specific tyrosine kinase to induce the correct formation of neuromuscular junctions. In postsynaptic membranes, there is a high-density pack of neurotransmitter receptors (Werle 2008; Wu et al. 2010).

A mature synapse contains a myelinated motor axon that divides into 20–100 unmyelinated terminal fibers (containing potassium and sodium channels). However, only one of these fibers innervates a single muscle fiber. These channels function in the control of the amplitudes and duration of action potentials. Opening of P/Q and possibly N-type calcium voltage-operated channels results in the release of acetylcholine from vesicles. In the membrane of muscle cells, the binding of two acetylcholine molecules causes a pore to open on the receptor, which allows entry of sodium, leading to muscle cell depolarization, propagation of action potentials across the muscle's surface, and muscle contraction (Hirsch 2007).

Thus, because it is the neuromuscular junction essential for coordinated movements, any interference with its function causes disorders. An example is the Isaacs syndrome, in which antibodies recognize K^+ channels in terminal nerve fibers and induce downregulation of expression of these proteins, causing visible muscular rippling movements, cramps, and stiffness (Scola et al. 1999).

2.7.2 Immunological Synapses

This concept became accepted in 1998 when images of interactions between T cells and antigen-presenting cells were published. However, the "immunological synapse" term dates back to the early 1980s. According to Dustin (2009), interaction is established by a synapse involving cells from the immune system, and consists of a ring of adhesion molecules surrounding a central cluster of antigen receptors. T lymphocytes are capable of organizing an immunological synapse based on actin–myosin motility.

The cellular structure of an immunological synapse at the interface of a T cell and a cell expressing the appropriate peptide–MHC complex is subject to a broad rearrangement. A T cell, for instance, when interacting with a B cell reorganizes the microtubule organizing center from the far side of the T cell to a location underneath the synapse in a cytoskeletal rearrangement (Huppa and Davis 2003).

T cells are able to detect a single ligand on antigen-presenting cells, but about ten of them are required to increase and maintain calcium levels for T cell activation through nuclear localization of expression of nuclear factor of activated T cells (NFAT) in order to activate T-helper-cell-specific gene expression (Feske et al. 2001).

Spatial organization of molecules occurs on immunological synapses, as Monks et al. (1998) showed in 1998. T-cell receptors (TCRs) and the adhesion integrin LFA-1 were identified on cell interfaces in distinct areas called supramolecular activation complexes (SMACs). TCRs are localized in the central region of SMACs, together with cell adhesion molecules such as LFA- 1 in peripheral regions of SMACs and CD43 and CD45 in distal regions of SMACs.

However, what is the time course for the formation of a mature immunological synapse? In $CD4^+$ effector T cells, firstly there is an interaction with B cells, for example. After 5–30 min, the mature synapse is formed, remaining for hours (usually, 24–48 h); simultaneously, cytokine production and release occurs and

continuous TCR signals are produced for maintenance. If synapse dissolution occurs, cells undergo several divisions (proliferation). When cytotoxic T lymphocytes, but not $CD4^+$ effector T cells are involved, synapse formation occurs rapidly (3–5 min), and the cytotoxic effector mechanisms can be activated within a few minutes. Lytic granules are rapidly transported to the central region of SMACs and target cell death happens within 20–30 min (Huppa and Davis 2003).

2.7.3 Cell–Cell Signaling Contact

Cell–cell signaling contact involving ligand–transmembrane receptor interactions depends on contact between cells. That is what occurs with ephrin receptor (EphR), and Delta and Notch receptors. The example chosen focuses on ephrin (Eph)–EphR interaction.

EphRs are tyrosine kinase receptors activated by Eph. They can induce bidirectional signaling because once the ligand binds to the receptor, it activates itself to act also like a receptor. EphRs form the largest subgroup of the tyrosine kinase receptor family and are divided into two classes: EphA receptors (interacting with EphA ligands) and EphB receptors (interacting with EphB ligands). They regulate, for example, T-cell and TCR-mediated responses, as EphB6 receptors act in immune response regulation. Moreover, they control glucose homeostasis and insulin secretion (a function of EphA receptors) and participate in osteoblast–osteoclast communication in bone homeostasis (Edwards and Mundy 2008).

These receptors are related to cancer development and progression, affecting the growth, migration, and invasion of cancer cells in vitro, and tumor growth, invasiveness, angiogenesis, and metastasis in vivo (Pasquale 2010).

Another function of EphR, performed by EphB4, is to influence embryonic stem cell development. During the differentiation process of this pluripotent cell, EphB4 (determinant on vessel formation) modifies the expression of genes related to the characteristics of mesodermal tissues, but not the expression of neuroectoderm-associated genes (Wang et al. 2004).

2.8 Role of the ECM and Cell Junctions in Cancer Development

2.8.1 Origin of Cancer and Metastasis

Cancer belongs to a category of diseases that arise as a result of genetic alterations, leading to accumulation of mutations and genetic instabilities, besides epigenetic alterations that together affect cell growth and death cycles. In humans, more than 90 % of all tumors are carcinomas (derived from epithelium tissue), although among

children, carcinomas are very rare, and instead leukemias, central nervous system cancer, lymphomas, sarcomas (origin in connective tissue), and embryonic cancers (teratomas) are a greater problem (Ries et al. 2006; González-Mariscal et al. 2007).

Tumor development and metastasis may fundamentally be attributed to genetic alterations in the genome of cancer cells. Such genetic changes include mutations located in proto-oncogenes and tumor suppressor genes, which then give rise to clones of tumor cells with different behavioral characteristics compared with their regular complements. These genetic alterations lead to deregulated expression profiles on components of the ECM and cell junctions. In general, cell dissociation, basal lamina invasion (by upregulation of lytic enzymes), and degradation of the ECM occurs (White and Muller 2007).

Progression of metastasis and invasion of cancer cells need these cells to modify their ability to adhere to both neighboring cells and the ECM. Cellular interactions with the ECM through integrins, besides other CAMs, play crucial roles in many aspects of tumor initiation and progression, (Drivalos et al. 2011).

2.8.2 Participation of Cadherins and Catenins

The cadherin adhesion complex needs to be intact and functional. The normal expression and function of E-cadherin as well as each catenin is critical for the normal state of cells. Absence or reduced expression of E-cadherin in cancer cell leads to invasiveness originating from cultured human carcinoma cells such as bladder, breast, lung, and pancreas tissue. Later, invasion of E-cadherin-positive cells was shown to be a result of deficiencies in various catenins. Thus, alterations in the invasion suppressor function of the E-cadherin–catenin complex may occur via different components of the catenin complex (Bremnes et al. 2002b).

There is evidence for the essential role of E-cadherin and catenins in cell–cell adhesion, and by which mechanism their functions may be altered (Fahraeus et al. 1992). Cells without E-cadherin expression do not aggregate or adhere to each other. In cells expressing E-cadherin, neutralizing this molecule with an antibody, deletion or mutation of its encoding gene, or depletion of calcium causes cells to dissociate. Cells with normal E-cadherin expression, but deficient expression of one of the catenins, are not able to aggregate or adhere to each other. Deletion of the genes encoding the catenins or mutations of the catenin proteins result in cells not being able to aggregate and adhere; and mutation of the intracellular domain of E-cadherin, which mnakes possible interactions with the catenins, yields nonaggregating nonadhesive cells (Berx and van Roy 2001; Bremnes et al. 2002b; Fukuoka et al. 2007; Drivalos et al. 2011).

Alteration of the E-cadherin–catenin complex may be either reversible or irreversible, depending on genetic causes. Phosphorylation is the best documented way of posttranslational regulation of the E-cadherin–catenin complex. Tyrosine phosphorylation of β-catenin and γ-catenin will lead to dissociation of the adhesion complex, and subsequently induces tumor cell invasiveness. Such phosphorylation

happens by activating receptors for hepatocyte growth factor or epidermal growth factor (EGF). The EGF receptor (EGFR) interacts with the central region of β-catenin (Bremnes et al. 2002b; Zhang et al. 2012). The EGFR possess tyrosine kinase activity that is triggered by autophosphorylation on its binding to EGF; through this mechanism, ligand binding to EGFR induces immediate phosphorylation of γ-catenin and β-catenin.

Some studies demonstrated that abnormal expression of E-cadherin, α-catenin, β-catenin, γ-catenin, and p120 catenin is associated with histological and pathological changes of tumor characteristics (differentiation, invasiveness, and vessel permeation), tumor stage, regional and distant metastasis, disease reversal, and survival (Pignatelli et al. 1994). More recently, associations between cadherin/catenin expression and clinicopathological features were also described in lung carcinoma patients (Kimura et al. 2000).

The E-cadherin–catenin complex serves as a cell–cell adhesion complex and in part as an intracellular signal transducer. The complex is upregulated and downregulated at various levels and by several agents, reversibly or permanently. From experimental data, we know that this complex has a cancer invasion suppressor function, and is frequently downregulated in malignant tumors. Moreover, data from clinical lung cancer studies have demonstrated a significant association between downregulation of this complex and clinical outcomes such as disease progression and shortened survival.

2.8.3 The Role of Tight Junctions in Cancer Development

A decrease in the occurrence of tight junctions in cells is responsible for reducing paracellular resistance and for facilitating invasion of tumor cells through endothelial cell layers, mostly in pancreatic tumor, leading to metastasis. Altered expressions of claudin-1, claudin-2, claudin-3, and claudin-5 is associated with colon cancer, altered expression of claudin-3 is associated with prostate cancer, and altered expression of claudin-4 is associated with pancreatic cancer (González-Mariscal et al. 2007). The expression of claudins also varies according to the stage of carcinogenesis.

In well-differentiated carcinomas of the pancreas, high levels of claudin-4 are detected, whereas in undifferentiated and highly invasive tumors, claudin-4 is weakly expressed. Additionally, in highly invasive pancreatic cell lines that poorly express claudin-4, transfection of this claudin impairs their invasiveness and metastatic potential. The expression of certain claudins in carcinomas has a predictive value for the disease outcome. Hence, expression of claudin-10 in hepatocellular carcinomas predicts disease recurrence after curative hepatectomy, and moderate-to-strong claudin-4 staining is associated with decreased survival rate in gastric adenocarcinomas (Michl et al. 2003; Resnick et al. 2005).

2.8.4 Integrins and FAKs

FAK, an intracellular tyrosine kinase recruited to the positions of integrin in adherens junctions, acts as a major mediator of signal transduction by cell surface receptors such as integrins, growth factors, and cytokine receptors. FAK plays key roles in the regulation of adhesion, cell dispersion, invasion, survival, proliferation, differentiation, and angiogenesis, events which are all involved in the development and progression of tumors. FAK can be decisive in cancer initiation, progression, and metastasis (mainly in breast tumors) (White and Muller 2007; Luo and Guan 2010).

FAK may play key roles in promoting tumorigenesis and metastasis, and it may serve as a critical target in the eradication of various cancers, including those of the mammary gland (Parsons 2003). In the initial stages of cancer development from mammary stem cells, some studies detected that there is an increased expression of FAK, and subsequent increase of expression of α_6 and β_1 integrins. The increase of these proteins is capable of turning normal stem cells into tumors, thus suggesting that FAK is a possible therapeutic target in breast cancer (Luo and Guan 2010) and its role in other cancers must be studied.

2.9 Conclusions and Perspectives

Cells are joined by cell junctions, structures greatly specialized with several functions; the main functions are anchorage, impermeability (and/or semipermeability), cell polarity, and communication, and junctions can also be part of a signaling pathway. To maintain the normal state of cells, the expression of all junction components is required. Junction complexes are found in all tissues with different compositions, according to cell specialization. In abnormal conditions, in which cells have lost their proliferative and systemic control, forming tumors, the participation of cell junctions is determinant. At this moment, an altered profile expression of junction proteins is configured, and cells become invasive, characteristic of cancer progression. Nowadays, numerous roles of cell junctions in cancer are known, but many others remain unclear.

The role of tight junctions in tumorigenic processes is multifaceted and involves several tactics affecting gene expression, cell proliferation, and the state of cell differentiation. Since the loss of cell–cell adhesion is a crucial step undertaken in transition regions of epithelial–mesenchymal tissues, strategies designed to overcome the altered expression of tight junction proteins found in tumors could eventually lead to therapies for treating and preventing cancers. Therefore, it is of utmost importance to understand the biological processes that control the expression of tight junction proteins, such as the regulation of their transcription. The E-cadherin–catenin complex is attractive for molecular target therapy. Screening to detect early-stage lung cancer and preneoplastic changes in high-risk populations of long-term

smokers may be a possible strategy for such therapy. In breast cancer, FAKs could be a candidate for therapy (Bremnes et al. 2002b).

An altered expression profile is an alternative molecular marker of cancer progression, besides being a therapeutic target. Knowledge of the normal state and neoplasic state of cell junctions is an important aspect for improvement of therapeutic methods and could also lead to new methods based on the findings of the studies reported here.

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Chapter 3
The Embryonic Rest Hypothesis of Cancer Development: 150 Years Later

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Abstract More than 150 years ago, Rudolf Virchow and Julius Cohnheim proposed the intriguing hypothesis that cancer may develop from embryonic cell remnants that remain in the developing organs following embryogenesis. This hypothesis, known as the "embryonic rest hypothesis of cancer development," was popular in textbooks of pathology in the ninetieth and twentieth centuries. At that time, the concept of stem cells was still unknown, and thus it was not clear what types of cells could be responsible for tumor occurrence. However, what was clear at that time was that the morphology of several tumors mimics developmentally early tissues. Today we know that some tumors express early developmental markers characteristic of embryonic cells, which could reflect either the epigenetic dedifferentiation of the somatic cells in which cancer develops to the state of early embryonic cells or that cancer originates in primitive stem cells closely related to the epiblast/germline. The identification of primitive epiblast/germline-derived very small embryonic/epiblast-like stem cells in several adult organs raised the possibility that cancer originates in these rare cells. Thus, very small embryonic/epiblast-like stem cells could be a missing link supporting this more than 150-year-old concept; however, further experimental evidence is needed to prove this tempting hypothesis.

Keywords Very small embryonic/epiblast-like stem cells • CXCR4 • Oct-4 • Cancer/testis antigens • Cancer • Germline

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3.1 Introduction

There are several mechanisms leading to cancer development. However, it is not completely clear at present whether cancer originates in mutated differentiated somatic cells or in the mutated cells from the stem/progenitor cell compartment. Nevertheless, it is widely accepted that organ/tissue regeneration and cancer development are likely to be closely related processes (Dvorak 1986; Beachy et al. 2004; Vakkila and Lotze 2004). Carcinogenesis is very often a response to chronic irritation, inflammation, and tissue damage, potentially developing through misappropriation of homeostatic mechanisms that govern tissue repair and stem cell self-renewal (Vakkila and Lotze 2004). Indeed, cancer incidence increases in association with chronic injury. These observations strongly support the involvement of tissue repair in the development of cancer, which suggests a role for stem cells in cancer origination (Reya et al. 2001).

The hypothesis that cancer develops in the stem/progenitor cell compartment is based on the assumption that self-renewing stem cells residing in organs and tissues, and not mature differentiated somatic cells such as those lining the bronchial or stomach mucosa (Houghton et al. 2004; Kim et al. 2005), may acquire and accumulate mutations during life. These mutations would be maintained in the stem cell compartment, and these self-renewing cells may then acquire additional mutations and epigenetic changes so that the genome is destabilized; at a certain point, an uncontrolled neoplastic proliferation may be initiated.

Indeed, recent evidence suggests that malignancy arises from the accumulation of mutations and maturation arrest of normal stem/progenitor cells rather than by the dedifferentiation of already differentiated cells (Bonnet and Dick 1997; Reya et al. 2001; Singh et al. 2004). Thus, normal stem cells may acquire mutations and give rise to cancer stem cells, which are subsequently responsible for tumor growth, tumor regrowth after unsuccessful radiochemotherapy, and establishing distant metastases. In fact, recent research has provided direct evidence that several neoplasms (e.g., brain tumors, prostate cancer, melanomas, colon cancer, and lung cancer) may in fact originate in the normal stem cell compartment (Reya et al. 2001; Welm et al. 2003; Fang et al. 2005; Kim et al. 2005). Specifically, rare populations of cells with stem cell properties have been identified within growing malignant tumors that, after inoculation of immunodeficient mice, are able to give rise to tumors that morphologically resemble those tumors from which they were initially purified (Ponti et al. 2005). However, it is still difficult to demonstrate such a phenomenon at the single-cell level, and usually a minimum number of cells have to be introduced to give rise to the tumor, suggesting the importance of cross talk between the primitive cells that initiate these malignancies.

Even if it is accepted that at least some malignancies originate from stem cells, it is unclear how developmentally primitive these cells of origin are. It is well known that the stem cell compartment displays a developmental hierarchy. However, although the common feature of stem cells is their ability to self-renew, it is widely accepted that different stem cells have different potentials for

differentiation into adult tissues. Besides tissue-committed stem cells (TCSCs; e.g., hematopoietic stem cells, muscle stem cells, or epidermal stem cells), which give rise to the cells present in a given tissue, evidence has accumulated that more primitive stem cells with a broader ability to differentiate also exist. Such cells with the capacity for multilineage differentiation have been described in adult tissues and have been given different names, such as multipotent adult stem cells (Beltrami et al. 2007), multipotent adult progenitor cells, marrow-isolated adult multilineage-inducible cells (D'Ippolito et al. 2004), and, recently identified by our team, so-called very small embryonic-like stem cells (VSELs) (Ratajczak et al. 2008a). The latter cells are particularly interesting because they express several markers characteristic of embryonic stem cells (Kucia et al. 2006).

The concept that adult tissues contain developmentally primitive cells with embryonic features that can lead to tumors is, surprisingly, not so novel. During the ninetieth and early twentieth centuries, several famous pathologists proposed that cancer may develop in populations of cells that are left in a dormant state in developing organs during embryogenesis. This so-called "embryonic rest hypothesis of cancer origin" was initially proposed by Recamier and Remak and was most extensively elaborated in its final form by Virchow, Durante, and Cohnheim, who was Virchow's pupil. It has been suggested that adult tissues contain embryonic remnants that normally lie dormant but that can be "activated" to become cancerous. In agreement with these theories, Wright proposed a germinal cell origin of Wilms tumor (nephroblastoma) and Beard postulated that tumors arise from displaced and activated trophoblasts or displaced germ cells. Nevertheless, at that time the putative cells responsible for these effects were neither clearly identified nor purified from adult tissues. Furthermore, since the term "stem cell" was not used in scientific language at that time, it is not clear to which specific type of cells these early pathologists were referring. However, it is reasonable to assume that they believed that such cells would be endowed with properties similar to those of stem cell as we understand them today.

In this chapter, we present evidence regarding (1) the existence of a developmentally primitive population of so-called VSELs deposited in early-developing tissues during organogenesis, (2) the developmental relationship of VSELs to the epiblast/germ lineage, and (3) some crucial epigenetic mechanisms based on silencing of imprinted genes involved in insulin-like growth factor (IGF) signaling that control VSEL proliferation and prevent "unleashed" expansion of these cells.

Based on the presence of VSELs in adult tissues, these cells could be the missing link that reconciles the embryonic rest hypothesis of cancer origin presented 100–150 years ago with current theories postulating cancer as a stem cell disorder. The hypothesis presented in this chapter, however, needs further experimental support and in the present form provides a basis for future experimentation. Our goal is to encourage other colleagues to consider the possibility that VSELs and other types of developmentally primitive stem cells deposited in adult tissues, such as multipotent adult stem cells or multipotent adult progenitor cells, could be involved in cancerogenesis.

3.2 The Germline as the Origin and "Scaffold" of the Stem Cell System in the Adult Body

Recently identified, VSELs express several markers characteristic of the germline (Shin et al. 2010; Zuba-Surma et al. 2008). From the developmental point of view, the germ lineage is immortal, as it passes genomic and mitochondrial DNA to the next generation. During embryogenesis, the germline creates mortal somata that are essential for the germline to fulfill its reproductive mission. The most primitive cell in the germline and the cell at the top of the germline hierarchy is the zygote, a result of fusion of two gametes—the oocyte and sperm—during fertilization. The germline potential is subsequently maintained during the first steps of development in the blastomeres of the morula and in the inner cell mass (ICM) of the blastocyst. At the level of the blastocyst, however, a portion of the cells surrounding the blastula "bud out" from the germline lineage and differentiate into the trophoblast, giving rise to the placenta. However, the rest of the cells in the ICM retain their germline character, and, after implantation of the blastocyst in the uterus, the ICM cells give rise to the epiblast. Pluripotent stem cells (PSCs) forming the epiblast also retain germline potential, for in the proximal epiblast, primordial germ cells (PGCs) become specified, and migrate to the genital ridges and give rise to the precursors of gametes. At the beginning of gastrulation, the other epiblast PSCs become specified into multipotent stem cells for mesoderm, ectoderm, and endoderm, which later give rise to unipotent TCSCs for all the developing organs and tissues in the embryo proper. We hypothesize that some of the epiblast PSCs and cells closely related to PGCs survive as Oct-4^{+} pluripotent VSELs in peripheral tissues and organs, serving as a reserve population for TCSCs (Kucia et al. 2006).

3.3 Molecular Evidence Supporting Embryonic Developmental Deposition of VSELs in Adult Tissues and Their Pluripotent Character

Gene expression studies have revealed that VSELs do in fact express several epiblast and germline markers, which supports their origin from early epiblast -derived cells sharing several markers with migrating PGCs, and are deposited (as hypothesized) during development in adult tissues as precursors of TCSCs, thus playing a role in organ rejuvenation (Kucia et al. 2006; Zuba-Surma et al. 2008). In support of this notion, molecular analysis of murine bone-marrow-derived VSELs has revealed that these cells express several genes characteristic of epiblast stem cells (*Gbx2*, *Fgf5*, and *Nodal*) and germline specification (*Stella*, *Prdm14*, *Fragilis*, *Blimp1*, *Nanos3*, and *Dnd1*) (Shin et al. 2009). The concept that adult tissues contain populations of more primitive quiescent stem cells besides the more active TCSCs has been recently demonstrated for skin epidermis (Blanpain et al. 2007)

and ovarian surface epithelium (Parte et al. 2011) and has been postulated for other organs (Ratajczak et al. 2008b). We envision that these more primitive cells could correspond to quiescent populations of VSELs.

VSELs exhibit several criteria that would be expected if they are PSCs (Ratajczak et al. 2011a, b). Specifically, they show (1) highly undifferentiated morphology, (2) high euchromatin content in the nucleus, (3) a high nuclear-to-cytoplasm ratio, (4) expression of PSC markers, such as Oct-4, Nanog, and SSEA, (4) open chromatin at the Oct-4 and Nanog promoters, (5) bivalent domains, (6) female PSC reactivation of the X chromosome, and (7), in some expansion models, multilineage differentiation into cells from all three germ layers (mesoderm, ectoderm, and endoderm). However, unlike classic embryonic stem cells, VSELs do not complement blastocyst development and do not form teratomas in immunodeficient animals. To explain this discrepancy, we observed that VSELs, in a similar manner to late migratory PGCs, modify the methylation of imprinted genes, a mechanism preventing them from uncontrolled proliferation and explaining their quiescent state in adult tissues (Shin et al. 2009, 2010). This important epigenetic modification, which occurs in VSELs and is relevant to tumor formation, will be discussed later in this chapter.

3.4 Indirect Evidence Linking Some Cancers with the Epiblast/Germline

There are several pieces of evidence supporting the embryonic rest hypothesis of cancer development and the potential involvement of epiblast/germline cells in this process. The first is the existence of "classic" germline tumors such as seminomas, ovarian tumors, yolk sac tumors, mediastinal or brain germ cell tumors, teratomas, and teratocarcinomas. Second, in several types of cancer cells, expression of cancer/testis (C/T) antigens has been observed (approximately 40 have been identified), and these are encoded by genes that are normally expressed only in the human germline but are also sometimes expressed unexpectedly in various nongonadal tumor types (e.g., gastric, lung, liver, and bladder carcinomas as well as melanomas, medulloblastomas, pediatric sarcomas, and germinal tumors) (Simpson et al. 2005; Sigalotti et al. 2008). The third piece of evidence is the expression of embryonic markers, such as human chorionic gonadotropin and/or carcinoembryonic antigen, in the plasma of many cancer patients. Specifically, several types of cancer contain the beta subunit of human chorionic gonadotropin or its fragments and/or carcinoembryonic antigen, which are employed in cancer diagnostics. Finally, it is known that several solid tumors express the embryonic/germline transcription factor Oct-4. As widely reported, Oct-4, a marker of germline PSCs, is expressed in various tumor types (e.g., gastric, lung, bladder, and oral mucosa carcinomas and germinal tumors) (Cheng 2004).

This large body of indirect evidence suggests that some malignancies could develop from very primitive cells retained in adult tissues. There is also a parallel possibility that all the above-mentioned changes emerge in cancer cells because

of epigenetic reprogramming of somatic cells during malignant transformation. However, as demonstrated recently (Houghton et al. 2004), gastric malignancies in experimental animals develop from embryonic stem cells that reside in gastric mucosa.

We envision that most primitive anaplastic types of solid tumors develop from the most primitive cells (from a developmental point of view) and could well be VSELs. It is worth mentioning that VSELs express not only several markers characteristic of the epiblast/germline, but also highly express messenger RNAs for several C/T antigens (Ratajczak et al. 2009). However, direct evidence is still needed to show that VSELs or pluripotent/multipotent populations of stem cells related to them and residing in adult tissues are a source of malignancies. One of the important potential connections between tumorigenesis and VSELs is epigenetic modification at the *Igf2–H19* locus. Erasure of parental imprinting (EOI) at this locus keeps these cells quiescent in adult tissues, whereas reversal by loss of imprinting (LOI) occurs in some malignancies.

We envision that several types of tumors may develop from Oct-4$^+$ stem cells residing in adult tissues by different mechanisms. For example, teratomas and teratocarcinomas may originate from Oct-4$^+$ epiblast/germline cells residing in adult tissues as a result of persistent somatic imprinting, in particular at the *Igf2–H19* locus. Furthermore, germinal tumors, including germinomas, seminomas, teratomas, dermoid cyst, and hydatidiform mole, most likely develop from mutated cells left along the PGC migratory route from the primitive streak to the genital ridges. Another group of tumors potentially linked to developmentally early cells are pediatric sarcomas such as rhabdomyosarcoma, neuroblastoma, Ewing sarcoma, and Wilms tumor (nephroblastoma). Evidence has accumulated that these tumors develop because of epigenetic changes in imprinted genes (LOI) and develop in developmentally early cells. Finally, other malignancies developing, for example, in the stomach or lung may originate from mutations in circulating Oct-4$^+$ cells that are incorporated at the wrong time or in the wrong place (e.g., *Helicobacter pylori* induced stomach cancer or tobacco-smoke-damaged lung) or in Oct-4$^+$ cells residing locally in these organs.

Interestingly, carcinogenesis could be initiated by aneuploidies induced by cancerogenes, which unbalance gene expression and lead to the selection of aneuploid clones of transformed cells (Liu et al. 2006; Hernando 2008). In this context, the fusion of a cell that expresses embryonic markers with a somatic cell in a given organ stimulated, for example, by inflammation could lead to the first step of malignancy development, the creation of a heterokaryon. According to this mechanism, VSELs, as highly fusogenic cells, are potential fusion partners for somatic cells. In this scenario, VSELs could provide several transcripts characteristic of developmentally early cells (e.g., Oct-4 or Klf-4), whereas somatic cells could supply chromosomes with proper genomic imprinting. The formation of such heterokaryons could be a first step in the selection of immortal aneuploid cells. In support of this possibility, not only embryonic stem cells and VSELs but also other types of more differentiated stem cells may fuse with somatic cells (Liu et al. 2006; Hernando 2008).

3.5 Paternally Imprinted Genes as Guardians of the Quiescent State of PGCs and VSELs: With *Igf2-H19* as a Master Yin-Yang Locus

It is well known that erasure of genomic imprinting is the basic mechanism that prevents teratoma formation by primitive germline cells (Hajkova et al. 2002; Yamazaki et al. 2003). Overall, among the 3.0×10^4–3.5×10^4 genes in the mammalian genome, there are approximately 80 genes that are paternally imprinted and expressed from the maternal or paternal chromosome only. It is well known that imprinted genes play a crucial role in embryogenesis, fetal growth, the totipotential state of the zygote, and the pluripotency of developmentally early stem cells. Also very important is that imprinted genes prevent parthenogenetic growth of PGCs (Reik and Walter 2001; Delaval and Feil 2004; Pannetier and Feil 2007). According to the parent–offspring conflict theory, paternally expressed imprinted genes enhance embryo growth and maternally expressed genes inhibit cell proliferation and negatively affect the size of the offspring (Bartolomei and Ferguson-Smith 2011). Accordingly, although during pregnancy, the father, through proper expression of paternally imprinted genes, contributes to body size and muscle mass of the developing fetus and wants the mother to devote as much of her resources as possible to the growth of his offspring, the mother wants to conserve as much of her resources as possible for future births (without compromising the health of the fetus she is currently carrying) by epigenetic modulation of genes by maternal imprinting marks (Bartolomei and Ferguson-Smith 2011).

The expression of imprinted genes is regulated by the imposition of epigenetic marks by DNA methylation within differentially methylated regions (DMRs), which are CpG-rich *cis* elements within the imprinted gene loci (Reik and Walter 2001; Delaval and Feil 2004; Pannetier and Feil 2007). These epigenetic marks imposed on DMRs in the female germline act on the promoters of imprinted genes, which results in the heritable repression of the maternal chromosomes. By contrast, the imposition of epigenetic marks by methylation of the chromosomes in the male germline occurs not at the promoters but rather within the intergenic regions (e.g., within the DMR between the *Igf2* and *H19* genes). Imprinted genes, in general, are highly expressed during embryogenesis, and many of them are subsequently downregulated after birth. Most of their DMRs are methylated in maternally derived chromosomes, and the DMRs of only a few genes, including *Igf2–H19*, are methylated in paternally derived chromosomes (Reik and Walter 2001; Delaval and Feil 2004; Pannetier and Feil 2007).

Modification of genomic imprinting also plays a crucial role in maintaining the pool of PSCs residing in adult tissues. As mentioned above, adult murine tissues harbor a population of pluripotent VSELs, which are Oct-4$^+$SSEA-1$^+$Sca-1$^+$Lin$^-$CD45$^-$. The corresponding population of human VSELs, which are Oct-4$^+$SSEA-4$^+$CD133$^+$Lin$^-$CD45$^-$, has also been identified (Kucia et al. 2006, 2007). We hypothesize that these cells are deposited in adult tissues during early embryogenesis and serve as a backup population for TCSCs. It has been demonstrated that

VSELs freshly isolated from murine bone marrow, such as PGCs, erase the paternally methylated imprints at regulatory DMRs within the *Igf2-H19* and *Rasgrf1* loci. However, unlike PGCs, they also hypermethylate the maternally methylated imprints within the DMRs for the IGF-2 receptor (*Igf2R*), *Kcnq1–p57^{KIP2}*, and *Peg1* (Shin et al. 2009). Because paternally expressed imprinted genes (*Igf2* and *Rasgrf1*) enhance embryonic growth and maternally expressed genes (*H19*, *p57^{KIP2}*, and *Igf2R*) inhibit cell proliferation (Sasaki et al. 2000; Delaval and Feil 2004), the unique genomic imprinting pattern observed in VSELs demonstrates the growth-repressive influence of imprinted genes on these cells (Shin et al. 2009, 2010).

The most important locus for imprinted genes is the *Igf2–H19* locus, which governs embryogenesis, fetal growth, behavioral development, the totipotential state of the zygote, and the pluripotency of developmentally early stem cells (Bartolomei and Ferguson-Smith 2011; Reik and Walter 2001; Hajkova et al. 2002; Delaval and Feil 2004; Pannetier and Feil 2007). This tandem gene not only expresses IGF-2 protein but also downregulates expression of IGF-1 receptor and thus modulates signaling of both IGF-2 and IGF-1, which regulate many vital aspects of cell biology (Gabory et al 2010; Ratajczak et al. 2010, 2011a, b, 2012; Chao and D'Amore 2008). Specifically, although *Igf2* encodes IGF-2, which is an autocrine/paracrine mitogen, transcription of *H19* yields a noncoding messenger RNA that is a precursor of several microRNAs that negatively affect cell proliferation. One of these microRNAs (miR-675) is involved in downregulation of IGF-1 receptor (Keniry et al. 2012).

On the other hand, EOI, including erasure of methylation within the DMR of the *Igf2–H19* locus (Fig. 3.1b), is one of the mechanisms that plays, as mentioned above, an important role in regulating quiescence of PGCs and VSELs (Fu et al. 2004; Shin et al. 2009). This mechanism of erasure of imprinting prevents these developmentally early cells from uncontrolled proliferation and teratoma formation and may be involved in the regulation of life span (Kucia et al. 2013; Ratajczak et al. 2010). EOI at the *Igf2–H19* locus is one of the major factors preventing parthenogenetic development in mammals, and the biological importance of this locus is demonstrated by the additional steps necessary for creation of viable bimaternal mice derived from two female sets of chromosomes (Kono et al. 2004). These mice are created by combining two haploid nuclei, one from a nongrowing oocyte and the other from a fully growing oocyte, into a diploid bimaternal zygote. Since female chromosomes have unmethylated DMRs for the *Igf2–H19* locus, the crucial step is appropriate genetic modulation of the expression of *Igf2* from one of the maternally derived sets of chromosomes to emulate the situation in which the *Igf2*-expressing, father-derived chromosome is present in the cells (Kono et al. 2004).

By contrast, hypermethylation of the *Igf2–H19* locus on both chromosomes (termed LOI, in contrast to EOI) results in *Igf2* overexpression (Fig. 3.1c) and is observed as an epigenetic change in several types of cancer (e.g., rhabdomyosarcoma and nephroblastoma) in which overexpressed IGF-2 acts as an autocrine growth factor for tumor cells. The best example is Beckwith–Wiedemann syndrome, which is associated with developmental organomegaly and several pediatric sarcomas (Choufani et al. 2010; Casola et al. 1997; Eggenschwiler et al. 1997). It is likely that some of these sarcomas may develop in populations of VSELs affected by LOI.

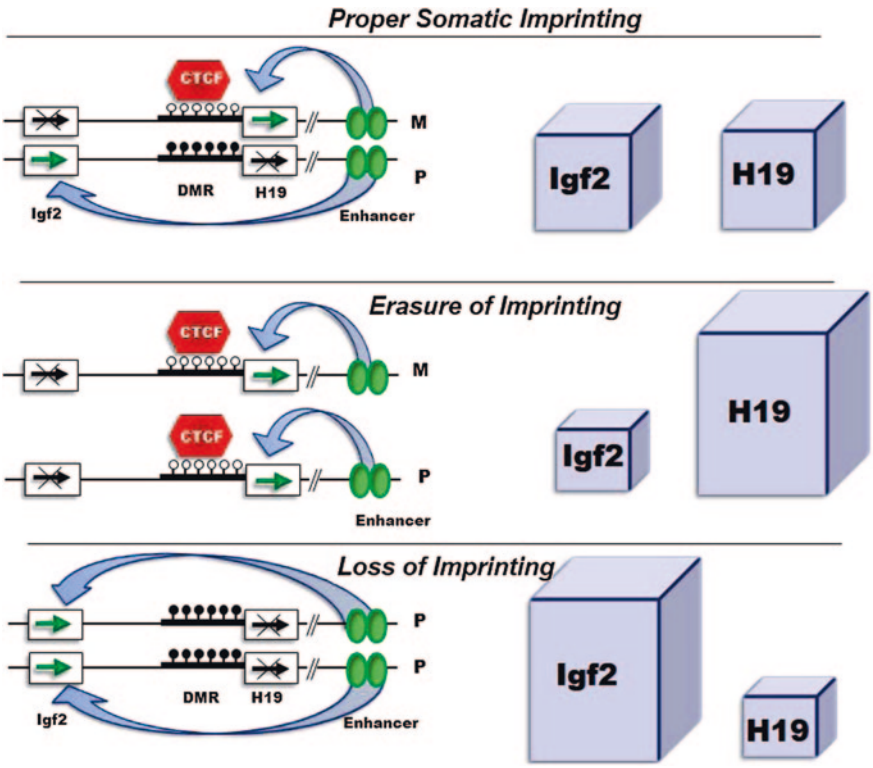

Fig. 3.1 Changes in the methylation state of differentially methylated regions (DMRs) and their impact on *Igf2* and *H19* expression. **a** Normal somatic imprinting at the *Igf2* and *H19* coding regions is separated by a DMR that is methylated (as shown by *filled lollypops*) on the paternal chromosome (*P*) and unmethylated (*open lollypops*) on the maternal chromosome (*M*). Expression of both genes is regulated by a 3′ distal enhancer depicted by two *green ellipses*. Methylation of the DMR on the paternal chromosome prevents binding of the CTCF insulator protein and allows activation of the *Igf2* promoter by the distal enhancer and transcription of *Igf2* messenger RNA (mRNA) from the paternal chromosome (*black arrow*). By contrast, since the DMR is unmethylated on the maternal chromosome, it binds CTCF, and this prevents activation of the *Igf2* promoter by the distal enhancer. As a result, only the *H19* noncoding RNA is transcribed from the maternal chromosome (*green arrow*). As the end result, the cell has a balanced expression of *Igf2* and *H19* from both chromosomes. **b** Erasure of imprinting at the *Igf2–H19* locus as seen in primordial germ cells and very small embryonic-like stem cells (VSELs) residing postdevelopmentally in adult tissues. DMRs on the paternal chromosome (*P*) and the maternal chromosome (*M*) are engaged by the CTCF insulator protein, and thus only the *H19* noncoding RNA is transcribed (*green arrows*) from the maternal and paternal chromosomes, contributing to the quiescent state of these cells (lacking autocrine insulin-like growth factor 2, IGF-2). **c** Loss of imprinting at the *Igf2–H19* locus as seen in tumor cells from several types of cancer (e.g., rhabdomyosarcoma, nephroblastoma, and gastrointestinal tumors). Since both DMRs are methylated, the insulator protein CTCF cannot bind to the DNA and the distal enhancer stimulates transcription of mRNA for IGF-2 from the maternal chromosome (*M*) and the paternal chromosome (*P*) (*green arrows*). Cells that have this epigenetic change are under autocrine IGF-2 stimulation

On the basis of this mechanism, the imprinted *Igf2–H19* tandem gene plays a pleiotropic role in several biological processes. Expression of this locus is tightly regulated by genomic imprinting, which ensures the balanced expression of both genes from either paternal or maternal chromosomes. Modification of expression at the *Igf2–H19* locus by EOI may have an important role in preventing malignant expansion of PGCs and VSELs, and, by contrast, LOI may lead to the development of malignancies. Thus, epigenetic changes within DMRs at the *Igf2–H19* locus may play an important role in the development of malignancies originating in epiblast/germline cells deposited during development in adult tissues. This mechanism, however, does not preclude involvement of other genetic aberrations that may affect this rare population of cells and lead them to malignant transformation.

3.6 Conclusions

There are several indications that some cancers originate in cells closely related to the germline. Our team recently identified a unique population of cells (VSELs) that express several epiblast/germline markers, implying that these rare cells could in fact be the source of several types of cancer. Accordingly, we assert that the Oct-4^{+} VSELs that express several germline markers (e.g., Oct-4, MvH, and C/T antigens) could be a population of stem cells that give rise to primitive tumors (e.g., teratomas, germinal tumors, and pediatric sarcomas) (Fig. 3.2).

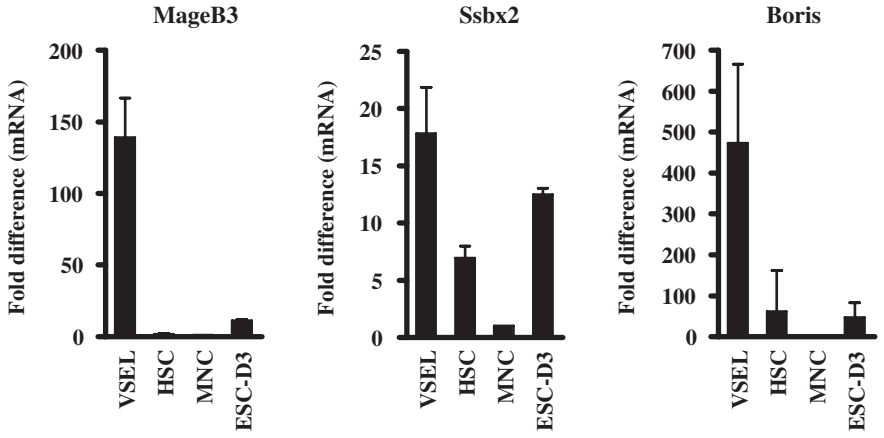

Fig. 3.2 Expression of cancer/testis (C/T) antigens by VSELs. Expression of selected C/T antigens encoded on X chromosome (MageB3, Ssbx2) and on non-X chromosome (BORIS) was evaluated in highly purified murine bone-marrow-derived VSELs, hematopoietic stem cells (*HSC*), bone marrow mononuclear cells (*MNC*), and an established murine embryonic stem cell line (ESC-D3) by employing real time reverse transcription PCR, using an ABI Prism 7000 sequence detection system. The relative expression level was calculated by the $2^{-\Delta\Delta Ct}$ method, using β_2-microglobulin as an endogenous housekeeping gene and mononuclear cells as a calibrator. Fold differences are shown as the mean ± the standard deviation from at least three independent experiments

Thus, the presence of VSELs in adult tissues may support the embryonic rest hypothesis or the germline origin hypothesis of cancer development and reconcile them with currently proposed stem cell theories of cancer development. In fact, markers present in VSELs, such as Oct-4, SSEA, and C/T antigens, have been identified in several types of cancer present in pediatric and adult patients. We envision that VSELs can initiate cancer by modifying imprinting (e.g., LOI), acquiring mutations, or fusing with other somatic cells. However, the potential involvement of VSELs in tumorigenesis requires further study and direct evidence.

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Chapter 4
Are Calcium Channels More Important Than Calcium Influx for Cell Proliferation?

Anne-Sophie Borowiec, Gabriel Bidaux and Thierry Capiod

Abstract Increases in both the basal cytosolic calcium concentration and cytosolic calcium transients play a major role in cell cycle progression, cell proliferation, and cell division. Calcium influx and calcium release from the endoplasmic reticulum are the major routes involved in the variations in cytosolic calcium concentration, and past studies have clearly shown that calcium influx controls cell growth and proliferation in several cell types. Furthermore, various studies in the past 30 years have demonstrated that cell-specific calcium channel expression levels are determinant in these physiological processes. Cell proliferation is directly linked to cell cycle progression, and it rapidly became evident that calcium channel expression interferes in this physiological process. It is also clear that the relationship between calcium influx and cell proliferation can be uncoupled in transformed and cancer cells, resulting in an external calcium-independent proliferation. Other divalent cations such as iron and zinc involved in cell proliferation permeating some calcium channels may interfere in this cellular process. Finally, we make the assumption that protein expression could be more important rather than channel function to trigger cell proliferation and that additional channel functions may be discovered soon.

Keywords Calcium channels • Cyclins • Cell proliferation • Mitosis • ORAI • SERCA • Transient receptor potential canonical • Cell cycle

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4.1 Introduction

Mammalian cells use calcium ions as a universal second messenger to modulate many cellular processes, including cell proliferation, development, migration, invasion, secretion, and learning and memory (Berridge et al. 2000, 2003; Carafoli 2002). Hormones, growth factors, cytokines, and neurotransmitters all elicit increases in intracellular calcium concentration, but differences in the temporal and spatial nature of the cytosolic calcium concentration transients enable a cell to adjust its response to a given stimulus (Berridge et al. 2003; Berridge 2009; Laude and Simpson 2009). Tremendous progress has been made in the last few decades in understanding key calcium-dependent pathways that regulate cell growth and division. The cell cycle consists of four primary phases: G_1 phase, the first gap phase; S phase, in which DNA synthesis occurs; G_2 phase, the second gap phase; and M phase, or mitosis, in which the chromosomes and cytoplasmic components are divided between two daughter cells (Fig. 4.1). The transitions between the four cell cycle phases are tightly regulated. Checkpoints during the cell cycle allow the cell to determine whether all is within the normal limits before proceeding to the next cell cycle phase (Hanahan and Weinberg 2011).

Intracellular second messengers, such as inositol 1,4,5-trisphosphate (InsP3), diacylglycerol, and arachidonic acid, are produced after phospholipase C and

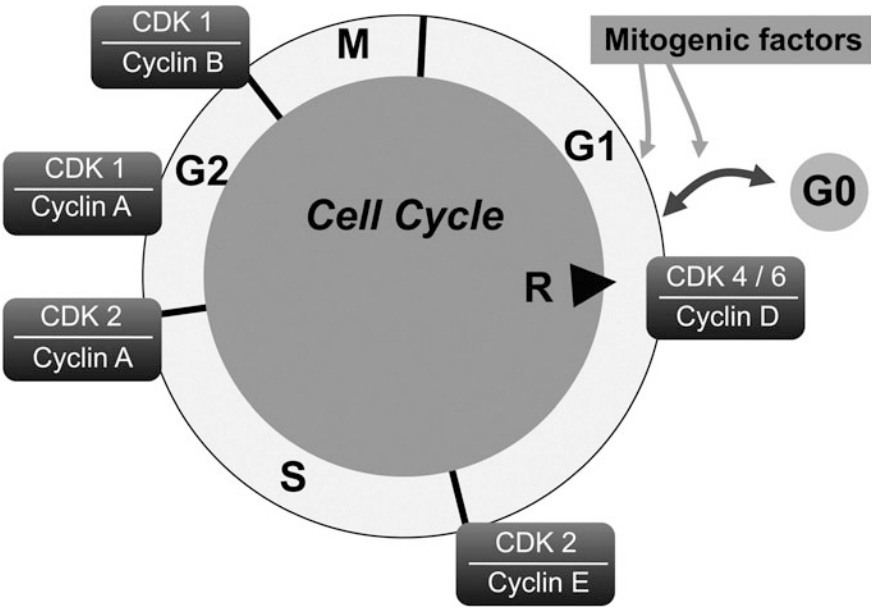

Fig. 4.1 Cell cycle phases and control by cyclins/cyclin-dependent kinases (*CDK*). The restriction point (*R*) is a point in G_1 of the animal cell cycle at which the cell becomes "committed" to the cell cycle, after which extracellular proliferation stimulants are no longer required

phospholipase A2 activation following plasma membrane G-protein-coupled receptor stimulation. InsP3-induced release of calcium from the endoplasmic reticulum (ER) leads to a decrease in the calcium concentration of the ER lumen (ER calcium contents), which in turn induces activation of plasma membrane calcium channels to generate a sustained influx of calcium (Berridge 2009). This type of calcium influx is called capacitative or store-operated calcium entry (SOCE) (Putney 1986; Parekh and Putney 2005). ER calcium content decreases below a threshold, triggering stromal interaction molecule 1 (STIM1) oligomerization, the first event of the sequence leading to SOCE activation (Stathopulos et al. 2006). It has been shown that oligomerization of STIM1 is followed by translocation of the multimers to membrane-adjacent ER areas where STIM1 can activate calcium influx (Stathopulos et al. 2006; Liou et al. 2007). Oligomerization of the isolated luminal domains of STIM1 is regulated by decreased luminal calcium binding to the EF hand with $K_{1/2}$ ~ 200–600 μM (Stathopulos et al. 2006). However, the whole process of calcium unbinding and oligomerization and redistribution of STIM1 has a calcium sensitivity of $K_{1/2}$ = 180–210 μM (Luik et al. 2006; Xu et al. 2006). STIM1 then binds to ORAI1 tetramers to trigger calcium-release-activated calcium current (I_{CRAC}), the archetypal SOCE current (Peinelt et al. 2006; Soboloff et al. 2006). ORAI1 tetramers form the calcium channel with properties similar to those described for I_{CRAC} (Hoth and Penner 1992). STIM1 also binds to transient receptor potential canonical (TRPC) cationic channels, the role in which SOCE is still a subject of discussion (Pani et al. 2012). The other two second messengers, diacylglycerol (Gudermann et al. 2004) and arachidonic acid (Mignen and Shuttleworth 2000), directly activate another set of plasma membrane calcium channels independently of ER calcium depletion, and calcium entry is therefore named noncapacitative calcium entry (NCCE). The molecular nature of the arachidonic acid related calcium current (I_{arc}) has been established, and the current flows through a heteropentameric assembly of three ORAI1 subunits and two ORAI3 subunits (Mignen et al. 2009) and is thought to be activated at low agonist concentration when ER calcium depletion does not occur (Shuttleworth and Mignen 2003).

The present chapter is focusing on the role of calcium influx and calcium channels in cell proliferation and cell cycle progression. The question here is to discuss whether or not cells need calcium influx to proliferate and what this may imply in terms of additional role for calcium channels in the control of cell proliferation. Several other recent reviews have addressed this question demonstrating that this issue is still controversial (Courjaret and Machaca 2012; Smyth and Putney 2012).

4.2 Is Proliferation Always Dependent on External Calcium?

The requirement of calcium for cell growth and division can be questioned in all mammalian cells. Several studies in the 1970s and early 1980s clearly demonstrated that sensitivity to external calcium depends on the degree of transformation, with

neoplastic cells being less dependent on external calcium. Proliferating mouse or human fibroblasts incubated in media containing low calcium concentrations ceased cellular division and accumulated in G_1 (Boynton et al. 1976; Hazelton et al. 1979; Whitfield et al. 1979). In RBL-2H3 and WI-38 cells, proliferation was blocked when extracellular calcium concentration was lowered to 20–60 μM (Hazelton et al. 1979; Tani et al. 2007). In BALBc/3T3 fibroblasts, this G_1 arrest was reversible, and returning the extracellular calcium content to normal levels enabled cells to undergo DNA synthesis within a few hours (Boynton et al. 1976). Cells were most sensitive to the depletion of extracellular calcium at two points during the cell cycle: in early G_1 and near the G_1/S boundary (Boynton et al. 1977). When human fibroblasts were stimulated by growth factors, depletion of extracellular calcium at any time during the first 8 h after stimulation resulted in inhibition of DNA synthesis (Takuwa et al. 1992). At later times, depletion of extracellular calcium had no effect on the ability of the cells to enter S phase. The crossing of the G_1/S border, which is marked by the initiation of DNA synthesis, represents commitment to division into two complete cells. Beyond this critical point, no further external signals are required (Reddy 1994). Some studies suggest an absolute requirement for calcium influx and overall calcium/calmodulin control of all steps from the response to growth factors in G_1 phase to DNA replication in S phase (Takuwa et al. 1995). Again, these arrests due to low calcium concentration were fully reversible as cells continued to proliferate after the addition of normal calcium levels to the media.

This requirement for extracellular calcium in growth and proliferation is modulated by the degree of cellular transformation. Neoplastic or transformed cells continued to proliferate in calcium-deficient media (Boynton 1988; Whitfield 1992). In contrast to their normal counterparts, human fibroblasts transformed with simian virus 40 proliferated normally in very low extracellular calcium concentrations (Boynton et al. 1977; Takuwa et al. 1993). Examination of primary cells, preneoplastic cells, and neoplastic cells revealed a gradient for extracellular calcium levels, required for proliferation (Swierenga et al. 1978). Primary C3H mouse skin cells have reduced rates of DNA synthesis when extracellular calcium concentration is lowered to 0.05–0.1 mM, whereas preneoplastic C3H/10T1/2 and MCA-C3H/10T1/2 type I mouse fibroblasts required a reduction to 0.01 mM extracellular calcium to inhibit DNA synthesis. Finally, the neoplastic MCA-C3H/1-T1/2 type III fibroblasts continued to proliferate with very low extracellular calcium levels. The proliferative responses of liver tumor cell lines in low calcium conditions reflected their tumorigenic potential (Swierenga et al. 1978). Therefore, the dire need for extracellular calcium is ruled out during neoplastic transformation. It is not clear how this change in extracellular calcium dependence affects intracellular calcium-dependent pathways and calcium concentration in all cellular compartments, and how cells adapt their need for calcium through remodeling of channels, pumps, and exchanger expression and activity.

When needed, the optimum external calcium concentration for cell proliferation was between 0.5 and 1 mM in all cell types. It was not clear whether calcium entering the cell might directly activate calcium-dependent signaling pathways or modulate the frequency of cytosolic calcium transients to control the amplitude of cellular responses. Increasing the calcium influx from the external medium into the

cytosol of *Xenopus* oocytes is known to increase both the frequency and the propagation velocity of the calcium fronts (Girard and Clapham 1993). Indeed, cytosolic calcium transients are dependent on external calcium in the long term, suggesting the need for calcium influx for their maintenance (Shuttleworth and Mignen 2003; Putney and Bird 2008). However, at least in some nonexcitable cells, calcium influx is not required to drive the oscillations. This latter was demonstrated using a technique called "lanthanide insulation" (Bird and Putney 2005). Relatively high concentrations (millimolar concentrations) of lanthanides (La^{3+}, Gd^{3+}) effectively inhibit both calcium influx and calcium extrusion at the plasma membrane (Van Breemen et al. 1972). In the presence of these high lanthanide concentrations, calcium oscillations are maintained, even in the absence of extracellular calcium (Sneyd et al. 2004; Bird and Putney 2005). More recent evidence against a role for calcium influx in cytosolic calcium concentration oscillations came from experiments done in the presence of small interfering RNA (siRNA) against ORAI1 or ORAI3. Cytosolic calcium concentration oscillations were recorded even when ORAI1 (Wedel et al. 2007) and ORAI3 (Thompson and Shuttleworth 2011) expression was greatly reduced, suggesting that neither I_{CRAC} nor I_{arc} was needed to maintain this cellular response, at least on a short time scale.

In transformed cells, increasing external concentration induced a small decrease in cell proliferation, and we have preliminary data suggesting that this is also the case in HEK293, human hepatoma cell line Huh-7, and HeLa cells. These three cell lines were able to proliferate in the absence of external calcium for at least 3 days with no decrease in cell viability. Thus, it is likely that, in some cell types, calcium-dependent cellular responses are driven by the transreticular fluxes, with little effect on calcium influx. Moreover, there is no evidence for calcium oscillations in mammalian cells in the absence of G-protein-coupled receptor stimulation and we did not record any change in intracellular calcium concentration over a period of 6 h in HEK293 cells kept in complete culture medium containing 10 % fetal calf serum (unpublished personal data). However, the data were recorded in nonsynchronized cells and, therefore, we cannot rule out that oscillations could be recorded at some specific point during the cell cycle. For instance, calcium transients have been observed in late G_1 phase prior to the initiation, in G_2 phase before entry into the M phase, during mitosis between metaphase and anaphase, and during cytokinesis (Santella 1998) and just before nuclear envelope breakdown in sea urchin eggs (Steinhardt and Alderton 1988). However, it still remains unclear whether these calcium transients are dependent on external calcium, and hence on calcium influx.

4.3 Evidence for a Role of Calcium Influx in Cell Proliferation

One of the strong arguments in favor of a role of calcium influx in cell proliferation comes from the use of calcium channel blockers. Drugs used to block L- and T-type voltage-dependent, SOCE, or NCCE channels have potential

antiproliferative effects in several tissues. The list of blockers includes verapamil, diltiazem, nifedipine, amlodipine, mibefradil, TH-1177, 2-APB, SKF 96365, and carboxyamidotriazole (CAI) (Nel et al. 1986; Taylor and Simpson 1992; Chung et al. 1994; Haverstick et al. 2000; Enfissi et al. 2004; Panner and Wurster 2006; Taylor et al. 2008). Other I_{CRAC} blockers have been identified and were the subject of a recent review (Parekh 2010). However, none of these drugs are entirely selective for one type of channel, but they all emphasize the role of calcium channels in the control of cell proliferation.

Manipulating calcium channel expression is another approach to establish a need for external calcium for cell proliferation in some cell types (Fig. 4.2). This chapter is focused on transient receptor potential (TRP) and ORAI channels, SOCE (Putney 1986; Parekh and Putney 2005), and NCCE (Shuttleworth et al. 2007) with their respective archetypal I_{CRAC} (Hoth and Penner 1992) and I_{arc} (Mignen and Shuttleworth 2000), but it is clear that several other types of calcium channels also play a major role in this process of cell proliferation; for example, purinergic P2X receptors (Burnstock 2009; Burnstock and Kennedy 2011), NMDA ionotropic receptors (Nacher and McEwen 2006), and voltage-gated T-type channels (Lory et al. 2006; Panner and Wurster 2006), all subjects of recent reviews, are also involved in cell proliferation. Evidence for the involvement of cyclic-nucleotide-gated channels in cell proliferation is sparse, but a recent article suggested that blocking hyperpolarization-activated cyclic-nucleotide-gated channels in embryonic stem cells resulted in an increase in S phase and a decrease in G_1 phase (Lau et al. 2011).

Knocking down TRPC, TRP vanilloid (TRPV), TRP melastatin (TRPM), or ORAI expression induces a decrease in cell proliferation as well as calcium influx in almost all cases (Table 4.1), strongly suggesting that reduced calcium entry and cell proliferation are directly linked (Sweeney et al. 2002; Yu et al. 2003; Hanano et

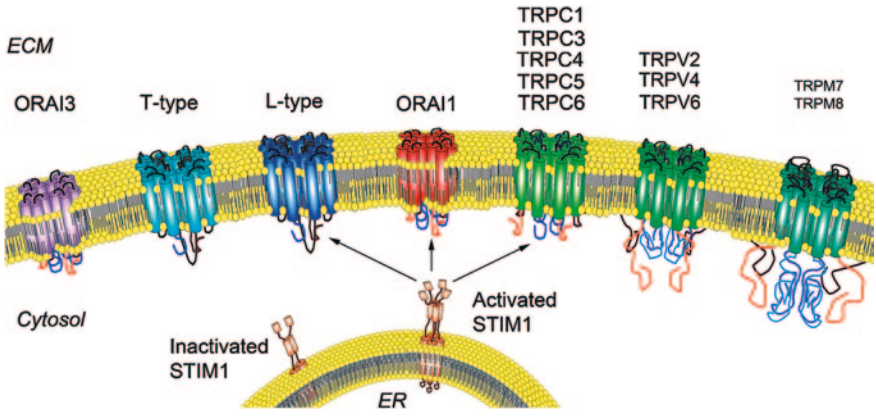

Fig. 4.2 Calcium channels playing a role in cell proliferation. Interactions between stromal interaction molecule 1 (*STIM1*) located on the endoplasmic reticulum (*ER*) membrane and L-type, ORAI1, and transient receptor potential canonical (*TRPC*) channels are indicated by the *arrows*. *TRPM* transient receptor potential melastatin, *TRPV* transient receptor potential vanilloid

Table 4.1 Summary of effects of increased transient receptor potential (TRP) and ORAI channel expression levels on cell proliferation rate

Channel	Increased proliferation	Decreased proliferation	In cell types
TRPC1	✓		Endothelial cells[a], rat neural stem cells, pulmonary artery cells, smooth muscle cells, and hippocampal neuronal cells (Sweeney et al. 2002; Wu et al. 2004; Beech 2005; Fiorio Pla et al. 2005; Abdullaev et al. 2008)
TRPC3	✓		EA.hy926 cells, hippocampal neuronal cells, skeletal muscle myoblasts, and human ovarian cancer cells (Wu et al. 2004; Yang et al. 2009a; Woo et al. 2010; Antigny et al. 2012)
TRPC4	✓		Endothelial cells[a], EA.hy926 cells, and human corneal epithelial cells (Yang et al. 2005; Abdullaev et al. 2008; Antigny et al. 2012)
TRPC5	✓		EA.hy926 cells (Antigny et al. 2012)
TRPC6	✓		Endothelial cells, pulmonary vascular smooth muscle cells, prostate cancer epithelial cells, esophageal carcinoma cells, human hepatoma Huh-7 cells, and human prostate cancer PC-3 and DU-145 cells (Yu et al. 2003; Thebault et al. 2006; Abdullaev et al. 2008; El Boustany et al. 2008; Ge et al. 2009; Ding et al. 2010a; Wang et al. 2010)
		✓	Psoriatic keratinocytes (Leuner et al. 2011)
TRPV2	✓		Prostate cancer PC-3 cells (Monet et al. 2010)
		✓	Glioma cells (Nabissi et al. 2010)
TRPV4	✓		Human esophageal epithelial cells (Ueda et al. 2011)
		✓	Cystic cholangiocytes (Gradilone et al. 2010)
TRPV6	✓		Breast and prostate cancer cells, and HEK293 cells (Wissenbach et al. 2004; Schwarz et al. 2006; Bolanz et al. 2008)
TRPM7	✓		MCF-7 breast cancer cells, human retinoblastoma cells, and pancreatic epithelial cells (Hanano et al. 2004; Guilbert et al. 2009; Yee et al. 2011)
TRPM8	✓		Pancreatic adenocarcinoma cells (Yee et al. 2010)
		✓	Prostate cancer PC-3 cells (Yang et al. 2009b)
ORAI1	✓		Endothelial cells, HEK293 cells, and vascular smooth muscle cells (Abdullaev et al. 2008; Potier et al. 2009; El Boustany et al. 2010)
ORAI3	✓		MCF-7 breast cancer cells (Faouzi et al. 2011)

Overexpression, growth factor stimulation, and knockdown experiments are all included in this table, a decrease in cell proliferation rate in knockdown experiments being included as a faster rate in the presence of the channel.
TRPC transient receptor potential canonical, *TRPM* transient receptor potential melastatin, *TRPV* transient receptor potential vanilloid
[a]In the study by Abdullaev et al. (2008), TRPC1 and TRPC4 induced a clear decrease in cell proliferation (60–70 %), with no apparent change in the amplitude of store-operated calcium entry.

al. 2004; Wissenbach et al. 2004; Wu et al. 2004; Beech 2005; Fiorio Pla et al. 2005; Yang et al. 2005, 2009a, b; Schwarz et al. 2006; Thebault et al. 2006; Abdullaev et al. 2008; Bolanz et al. 2008; El Boustany et al. 2008, 2010; Ge et al. 2009; Guilbert et al. 2009; Potier et al. 2009; Ding et al. 2010a; Gradilone et al. 2010; Monet et al. 2010; Nabissi et al. 2010; Wang et al. 2010; Woo et al. 2010; Yee et al. 2010, 2011; Faouzi et al. 2011; Leuner et al. 2011; Ueda et al. 2011; Antigny et al. 2012).

However, there are a few exceptions, described below, to this scheme. Knocking down TRPC1 and TRPC4 decreased cell proliferation, with no effect on SOCE or I_{CRAC} activity in endothelial cells (Abdullaev et al. 2008). This is rather unexpected and could suggest an additional role for these channels. This point will be discussed later.

The behavior of TRPV4 is rather different from that of other TRP channels. Activation of TRPV4 by a synthetic phorbol derivative, 4α-phorbol 2,13-didecanoate, induced an increase in calcium influx and a decrease in proliferation of cholangiocytes in polycystic liver diseases (Gradilone et al. 2010) and human esophageal epithelial cells (Ueda et al. 2011). The last two sets of data provide arguments against a role of calcium influx in cell proliferation in some particular cases. In addition, knocking down STIM1, the ER plasma membrane counterpart of SOCE channels (Cahalan 2009; Varnai et al. 2009), has no effect on cell proliferation in vascular smooth muscle cells (Li et al. 2008), human myoblasts (Darbellay et al. 2009), HEK293 cells (El Boustany et al. 2010), the human umbilical vein endothelial cell (HUVEC) derived cell line EA.hy926 (Antigny et al. 2012), and probably normal HUVECs (Abdullaev et al. 2008). At the same time, I_{CRAC} is greatly reduced, which suggests strongly that, in some cells, this current and cell proliferation is uncoupled. Another exception to the direct relation between calcium influx and proliferation is the increase in psoriatic keratinocyte proliferation, coupled to reduced TRPC6 expression (Leuner et al. 2011). The results for TRPM8 are controversial. TRPM8 is needed for pancreatic adenocarcinoma proliferation (Yee et al. 2010) but TRPM8 overexpression could reduce proliferation of PC-3 human prostate cancer cells (Yang et al. 2009b), although the use of rat DNA in these experiments could be questioned.

Changes in calcium channel expression are observed in several diseases. To the same extent, growth factors induce exit from quiescence, cell proliferation, and expression of calcium channels. Growth factors are indeed very important for cell proliferation, and it was shown that calcium channel expression was upregulated, probably to provide the cells with the calcium they need to progress in the cell cycle and divide (Golovina et al. 2001; Yu et al. 2003; Fiorio Pla et al. 2005; El Boustany et al. 2008; El Hiani et al. 2009; Ge et al. 2009; Wang et al. 2010; Davis et al. 2012; Ogawa et al. 2012; Tajeddine and Gailly 2012) (Fig. 4.3).

However, direct evidence connecting calcium channel overexpression and cell proliferation is rare. TRPC6 overexpression increased both SOCE amplitude and cell proliferation rate in human hepatoma cells (El Boustany et al. 2008). Overexpression of ORAI1 alone reduces the amplitude of the native I_{CRAC} (Peinelt et al. 2006; Soboloff et al. 2006), resulting in a clear and expected decrease in cell proliferation rate in A549 lung cancer cells (Hou et al. 2011).

Several reviews have focused on calcium channel expression in various diseases, involving physiopathological cell proliferation. For instance, TRP channels are deeply involved in cancer (Bodding 2007; Gkika and Prevarskaya 2009; Ding et al. 2010a; Santoni et al. 2011), and ORAI1 is involved in cancer (Roberts-Thomson et al. 2010) and immune responses (Feske 2010; Feske et al. 2010).

4.4 Calcium Channels and Cell Cycle Distribution

The need for calcium influx in cell proliferation implies that calcium channel expression is a key point in cell cycle progression. Recording voltage-gated calcium channels during the cell cycle revealed large variations in L-type and T-type currents (Kuga et al. 1996; Cribbs 2006), and similar behavior was later observed for SOCE activity (Bodding 2001; Tani et al. 2007). It was then obvious that a decrease in calcium channel expression would lead to blocking of the cell cycle, and the data

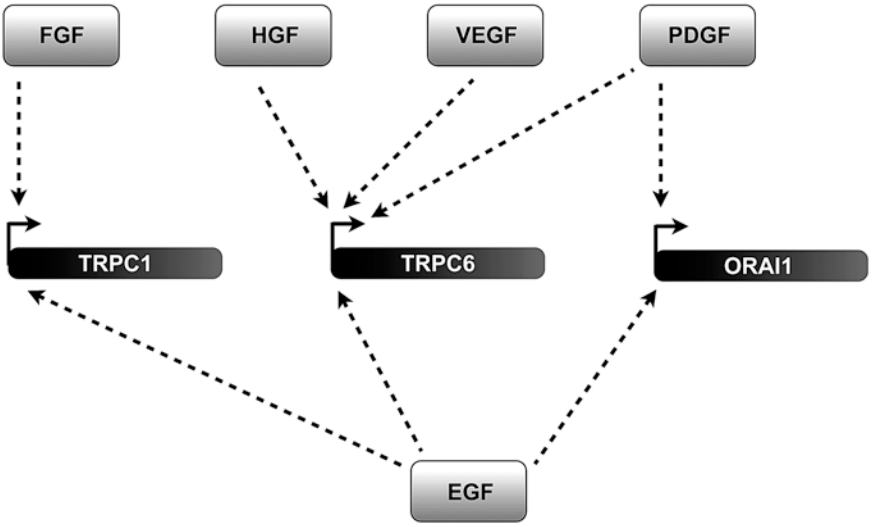

Fig. 4.3 TRPC1 expression is upregulated by fibroblast growth factor (*FGF*) in rat neural stem cells (Fiorio Pla et al. 2005) and epidermal growth factor (*EGF*) in MCF-7 human breast cancer cells (El Hiani et al. 2009) and non-small-cell lung carcinoma cells (Tajeddine and Gailly 2012). TRPC6 expression is upregulated by platelet-derived growth factor (*PDGF*) in smooth muscle cells (Yu et al. 2003), vascular endothelial growth factor (*VEGF*) in human umbilical vein endothelial cells (Ge et al. 2009), hepatocyte growth factor (*HGF*) in human hepatoma cells (El Boustany et al. 2008) and DU-145 and PC-3 human prostate cancer cells (Wang et al. 2010), and EGF in human hepatoma cells (El Boustany et al. 2008). and ORAI1 expression is upregulated by PDGF in human pulmonary artery smooth muscle cells (Ogawa et al. 2012) and EGF in MDA-MB-468 breast cancer cells (Davis et al. 2012). Serum induces expression of TRPC6 (El Boustany et al. 2008) and TRPC1 (Golovina et al. 2001) in human hepatoma cells and human pulmonary artery myocytes, respectively

Table 4.2 Cell cycle block and calcium channel expression

TRP channel	Knocked down	Overexpression	Consequences for the cell cycle (in cell types)
TRPC1	✓		G_1 block (endothelial progenitor cells) (Kuang et al. 2011)
TRPC3	✓		Prolonged M phase (human ovarian cancer cells) (Yang et al. 2009a)
TRPC6	✓		G_2/M block (PC-3 and D-U145 human prostate cancer cells, human gastric cancer cells, esophageal carcinoma cells, human glioma cells, and human esophageal cancer cells) (Cai et al. 2009; Ge et al. 2009; Shi et al. 2009; Ding et al. 2010a, b; Wang et al. 2010)
TRPM7	✓		G_0/G_1 block (pancreatic epithelial cells) (Yee et al. 2011); slow G_1/S progression (human retinoblastoma cells) (Hanano et al. 2004)
TRPM8	✓		G_0/G_1 increase and G_2/M decrease (pancreatic adenocarcinoma cells) (Yee et al. 2010)
ORAI1	✓		G_1 decrease and G_2/M increase (endothelial cells and HEK293 cells) (Abdullaev et al. 2008; El Boustany et al. 2010)
		✓	G_0/G_1 increase and S decrease (PC-3 prostate cancer cells) (Yang et al. 2009b)
ORAI3	✓		G_1 block and S and G_2/M decrease (MCF-7 and T47D human breast cancer cells; no effect in MCF-10A cells) (Faouzi et al. 2011)
		✓	G_0/G_1 block (A549 lung cancer cells) (Hou et al. 2011)

obtained with TRP and ORAI channels are complied in Table 4.2 (Hanano et al. 2004; Abdullaev et al. 2008; Cai et al. 2009; Ge et al. 2009; Shi et al. 2009; Yang et al. 2009a, b; Ding et al. 2010a, b; El Boustany et al. 2010; Wang et al. 2010; Yee et al. 2010, 2011; Faouzi et al. 2011; Hou et al. 2011; Kuang et al. 2011).

Undoubtedly, the level of calcium channel expression plays a major role in the relation between calcium influx and cell proliferation by modulating the amount of calcium flowing into the cell. Several channels (but not all of them) have been directly related to this process, but only a few studies have investigated the consequences of extinction of calcium channels on cell cycle distribution. However, overexpression of T-type calcium channels resulted in controversial effects, as it induced a clear increase in cytosolic calcium concentration without affecting cell

proliferation (Chemin et al. 2000), strongly suggesting decoupling between the two physiological responses in HEK293 cells.

In HUVEC and HEK293 cells, knocking down ORAI1 channels resulted in a dramatic reduction in cell proliferation (approximately 70 %) with a small increase in G_2/M phase (with a parallel decrease in G_1) of about 10 % (Abdullaev et al. 2008; El Boustany et al. 2010). TRPC6 has been extensively studied, and all data are in favor of a block in G_2/M phase. All studies but one showed a large decrease in cell proliferation (Cai et al. 2009; Shi et al. 2009; Wang et al. 2010). This was associated with a small but significant increase of G_2/M phase of about 10–20 %. The remaining study showed only a small decrease in cell proliferation and a small increase in G_2/M phase (Ding et al. 2010b).

Knocking down TRPC1 induced a complete block in G_1 phase, with a 50 % decrease in endothelial progenitor cell proliferation (Kuang et al. 2011) within 48 h. However, in this study, cells were first starved of serum for 24 h and then transfected with siRNA against TRPC1. G_1 block and inhibition of cell proliferation were also observed in MCF-7 cells after downregulation of ORAI3 by siRNA, but this was associated with a large decrease in cell viability (Faouzi et al. 2011).

To summarize, knocking down calcium channel expression often has a more dramatic effect on inhibition of cell proliferation than on cell cycle block. Thus, in this particular case, an increase in the number of cells in one specific phase would reflect a bottleneck more than a real block.

The block in G_2/M phase observed in ORAI1 and TRPC6 knockdown is interesting as there is evidence that SOCE is inactivated during M phase. The first suggestion that calcium influx is inhibited during cell division was reported more than 20 years ago in a study of HeLa cells (Volpi and Berlin 1988), probably through uncoupling of store depletion from SOCE (Preston et al. 1991). This was confirmed recently in HEK293, Cos-7, RBL-2H3, and HeLa cells, where SOCE is dramatically reduced during mitosis (Tani et al. 2007; Russa et al. 2008; Smyth et al. 2009; El Boustany et al. 2010). STIM1 phosphorylation (Smyth et al. 2009), decrease in ORAI1 expression (El Boustany et al. 2010), and internalization of the ORAI1 channel (Yu et al. 2009, 2010) during mitosis were recently suggested to be the reasons for the observed decrease in SOCE. The significance of this has already been discussed (Arredouani et al. 2010) and involves microtubule-network remodeling (Russa et al. 2008) as perturbation of the actin cytoskeleton resulted in a decrease in SOCE amplitude (Patterson et al. 1999).

4.5 Cell Cycle Block and Store-Operated Calcium Entry

Another way to study the relation between SOCE and cell proliferation is to block the cell cycle using drugs and serum deprival. With use of this method, it became possible to record calcium current with a high yield of cells blocked in one particular stage of the cell cycle. However, it is clear that cell cycle block has several other effects on calcium signaling proteins, in particular on ER calcium contents. Serum-free culture and medium containing a low level of serum are well-known methods

for blocking the cell cycle in G_1 phase, resulting in a large increase, up to 90 %, in cells blocked in this phase. This block induced a clear reduction in SOCE amplitude in keratinocytes (Leuner et al. 2011), human hepatoma cells (El Boustany et al. 2008), human pulmonary artery myocytes (Golovina et al. 2001), and RBL cells (Bodding 2001; Tani et al. 2007). Block in S phase is usually achieved using hydroxyurea, again resulting in SOCE inhibition of about 60 %. However, no more than 50 % of the cells were found in S phase using this method (El Boustany et al. 2010). Block in G_2 and M phases can be achieved by several means. Up to 70 % of RBL-2H3 cells were synchronized in G_2 phase using the topoisomerase II inhibitor HOECHST 33342 (Tani et al. 2007), and up 90 % of HeLa cells (Vassilev et al. 2006) and HEK293 cells (El Boustany et al. 2010) were synchronized in G_2/M phase using RO-33O6, a reversible cyclin-dependent kinase (CDK) 1 inhibitor. Again, this resulted in a dramatic decrease in SOCE in all cases. Another way to synchronize cells in mitosis is to treat them with nocodazole. Mitosis was assessed on the basis of spherical morphology, and in these conditions SOCE was absent in the nocodazole-treated mitotic HeLa cells (Smyth et al. 2009). It is, therefore, clear that the amount of calcium entering the cells is strictly adaptable to the cell's needs.

4.6 Amplitude of Calcium Inward Currents and the Cell Cycle

There is no direct evidence that calcium channel expression varies during the cell cycle. Indeed, there are several studies demonstrating that the amplitudes of inward currents are different from one cell cycle phase to another, but this may reflect regulation rather than expression (Fig. 4.4). Voltage-dependent L-type calcium currents were measured throughout the entire cell cycle with a peak in G_1 phase, whereas T-type currents were measured in S phase only in primary culture of rat aortic smooth

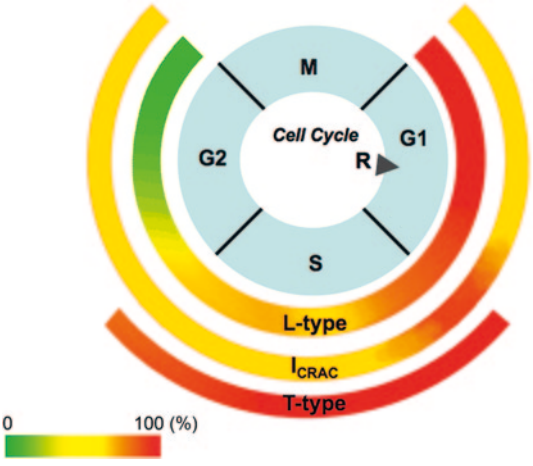

Fig. 4.4 Calcium influx intensities during the cell cycle for voltage-gated T-type, L-type, and I_{CRAC} calcium channels according to Kuga et al. (1996) and Tani et al. (2007)

muscle cells (Kuga et al. 1996). It was also shown that I_{CRAC} densities increase by 50 % at the transition between G_1 phase and S phase and remain elevated throughout S and G_2 phase in comparison with normal levels in G_1 phase, before a dramatic 70 % fall during metaphase of mitosis (Tani et al. 2007). In this study, cells were released from cell cycle block and currents were measured afterward. This explains the apparent contradiction between the data obtained in this study and the data obtained in other studies where I_{CRAC} was reduced in all cell cycle phases, as mentioned in the previous section. Hence, cells get through the cell division phase without the need for SOCE (Machaca and Haun 2002) because of uncoupling of ER and plasma membrane channels (Preston et al. 1991), ORAI1 internalization during the cell division phase (Yu et al. 2009), or STIM1 phosphorylation (Smyth et al. 2009). There is some kind of contradiction between the observed inactivation of calcium influx during M phase of the cell cycle (Preston et al. 1991; Yu et al. 2009) and the block in G_2/M phase in ORAI1 knockdown (Abdullaev et al. 2008; El Boustany et al. 2010) and TRPC6 knockdown (Cai et al. 2009; Ge et al. 2009; Shi et al. 2009; Ding et al. 2010a, b; Wang et al. 2010). The presence of calcium channels should not be needed if mitosis can occur in the absence of calcium influx. However, it is possible that calcium is important for G_2 progression and that calcium influx is switched off during mitosis only. The other possibility is that these calcium channels have additional functions in the cell cycle and mitosis. This issue requires further investigation.

4.7 Constitutive Calcium Entry and Membrane Potential

Evidence for SOCE and NCCE activity as well as an increase in cytosolic free calcium concentration during the cell cycle in mammalian cells is sparse if not nonexistent. In the absence of stimuli, these calcium entry pathways should not be activated unless some constitutive coupling exists. It has been known for years that the cytosolic free calcium concentration is decreased in the absence of external calcium (Berthon et al. 1984). As said in Sect. 4.1, SOCE activation is triggered by ER calcium depletion that is sensed by STIM1 and stromal interaction molecule 2 (STIM2). STIM2 activation is more sensitive to partial ER calcium depletion (Zheng et al. 2008; Stathopulos et al. 2009),as observed, for instance, in several diseases (Mekahli et al. 2011). STIM2 has been proposed as an alternative to STIM1 for ORAI1 activation (Oh-Hora et al. 2008; Darbellay et al. 2010). Moreover, cytosolic free calcium concentration in HeLa and HEK293 cells was lower in STIM2 knockdown but was not affected in STIM1 knockdown (Brandman et al. 2007). Therefore, membrane potential variations during the cell cycle could modulate the amplitude of a constitutive calcium entry in mammalian cells independently of SOCE or NCCE activation. However, constitutive SOCE activation resulting from partial depletion of ER calcium contents could explain why calcium channels blockers, such as CAI, 2-aminoethyldiphenyl borate, and SKF 96365, are able to block cell proliferation. It is, however, important to ensure that none of these blockers are highly specific. One of the first I_{CRAC} channel blockers that was used experimentally was the imidazole antimycotic SKF 96365 (Merritt et al. 1990). Although this

compound blocked SOCE in many cell types, its use was undermined by the finding that it blocked other ion channels with similar potencies (Franzius et al. 1994). 2-Aminoethyldiphenyl borate is another popular I_{CRAC} channel inhibitor, but has too many off-target actions to be used reliably (Putney 2010). CAI depolarizes mitochondria and therefore reduces the capacity of the organelles to buffer cytoplasmic calcium (Mignen et al. 2005), leading to greater calcium-dependent inactivation of the I_{CRAC} channels (Gilabert and Parekh 2000; Hoth et al. 2000).

TRPV6 channels are calcium-selective but are inactivated at resting levels of calcium. Lowering cytoplasmic free calcium concentration removes the calcium inhibition and therefore TRPV6 channels open (Vennekens et al. 2000). calcium influx follows, but this is not store-operated. It is not clear how cytoplasmic free calcium concentration would vary duting the cell cycle, and whether a single key regulator would be enough. calcium–Mg^{2+}-ATPase, Na^{+}/calcium exchangers, mitochondria buffering capacity, and membrane potential are among the mechanisms that would control cytoplasmic free calcium concentration. Further supporting evidence for a role of TRPV6 in constitutive calcium entry comes from experiments showing that the cytosolic free calcium concentration was increased by at least twofold in TRPV6-overexpressing HEK293 cells (Sternfeld et al. 2005).

Voltage-gated calcium channels may also account for constitutive calcium entry during the cell cycle. The unique low-voltage-dependent activation/inactivation and slow deactivation of T-type calcium channels indicate that these channels may play a physiological role in carrying depolarizing current at low membrane potentials. Therefore, these channels may play a direct role in regulating intracellular calcium concentration, especially in nonexcitable tissues, including some cancerous cells. At low voltages, T-type calcium channels are known to mediate a phenomenon known as “window current” (Crunelli et al. 2005). The term “window” refers to the voltage overlap between the activation and steady-state inactivation at low or resting membrane potentials. As a result, there is a sustained inward calcium current carried by a small portion of channels that are not completely inactivated. Window current allows T-type calcium channels to regulate calcium homeostasis under nonstimulated or resting membrane conditions (Bean and McDonough 1998). These T-type calcium channels are found on almost all cell types and therefore may have to adapt their modes of activation when expressed in different cell types. For example, lymphocytes, prostate cancer cells, and breast cancer cells are not known to spontaneously depolarize, but nevertheless they express functioning voltage-dependent calcium channels (Mariot et al. 2002; Kotturi et al. 2006; Taylor et al. 2008).

What is important here is the fact that membrane potential should modulate constitutive or activated calcium entry. Early studies reported a decrease in membrane potential in transformed cells (Tokuoka and Morioka 1957; Binggeli and Cameron 1980). Although this observation has stood the test of time, a simple relation between membrane potential and the cell cycle cannot be drawn for several reasons, mainly because channel expression varies from one cell type to another and within a single cell type during the different phases of the cell cycle. For example, in the same cell, hyperpolarization should increase the driving force for calcium ions, hence increasing the amplitude of voltage-independent calcium

channels such as I_{CRAC} or I_{arc}, while preventing activation of voltage-gated calcium channels (Capiod 2011). It is clear that membrane potential oscillates during the cell cycle (Blackiston et al. 2009; Becchetti 2011), and membrane potential is now examined as a key regulator of proliferation. G_1/S and G_2/M transitions require hyperpolarization and depolarization, respectively (Blackiston et al. 2009).

4.8 *Effects of Other Divalents and Ethylene Glycol Bis(2-aminoethyl ether)tetraacetic Acid Buffering*

Among the several other divalent cations, iron (Cazzola et al. 1990; Templeton and Liu 2003; Steegmann-Olmedillas 2011) and zinc (Beyersmann and Haase 2001; Dreosti 2001) are clearly involved in cell proliferation, whereas cadmium, nickel, and manganese induce cell death and apoptosis (Hirata 2002; Pulido and Parrish 2003). Iron is a metal vital for the maintenance of life and is an essential component of many proteins and enzymes involved in cell growth and division (Hershko 1994; Andrews 1999; Buss et al. 2004; Yu et al. 2007). Depletion of iron in cells typically results in G_1/S arrest and apoptosis (Brodie et al. 1993; Hileti et al. 1995; Lucas et al. 1995), indicating that this metal is essential for cell cycle progression, growth, and division (Kwok and Richardson 2002; Le and Richardson 2002). Under some conditions of iron deprivation, G_2/M arrest has also been identified (Renton and Jeitner 1996). Despite this, it is surprising that little is known about the role of iron in cell cycle regulation. Furthermore, it has become clear that some iron chelators show promising anticancer activity by inducing cell cycle arrest and apoptosis (Buss et al. 2004; Kalinowski and Richardson 2005). Deferoxamine and deferiprone have been used for decades to maintain safe levels of iron, firstly by preventing iron from reaching toxic levels, and secondly by removing excess iron when toxic levels have already been reached. They have antiproliferative effects in several cancer cell lines (Chenoufi et al. 1998a, b; Simonart et al. 2002; Okada et al. 2007), and these properties mean the chelators have therapeutic potential and could be used clinically (Kontoghiorghes et al. 2008; Hatcher et al. 2009).

The question is whether these divalent cations could flow into the cell via calcium channels. We have shown that some divalent cations are both blockers and permeators of P2X purinoceptors in guinea-pig hepatocytes (Capiod 1998). Iron permeates TRPC6 (Mwanjewe and Grover 2004), zinc permeates TRPV6 (Kovacs et al. 2011), TRPC6 (Gibon et al. 2011), and TRPM3 (Wagner et al. 2010), and cadmium permeates TRPV6 (Kovacs et al. 2011) and TRPM7 (Martineau et al. 2011). Interestingly, although Mn^{2+} also potently blocks I_{CRAC} (Hoth and Penner 1993), SOCE channels can be permeated by Mn^{2+}, suggesting that some of the blocking divalent metal ions may also permeate the channel (Fasolato et al. 1993).

Measuring cell proliferation in the absence of external calcium is often done in the presence of ethylene glycol bis(2-aminoethyl ether)tetraacetic acid to chelate residual calcium mainly present in the serum. Ethylene glycol bis(2-aminoethyl ether)tetraacetic acid has a zinc-binding affinity of 4.5×10^{-10} M (Lee

et al. 2008) and an iron-binding affinity of about 6.8×10^{-6} M for Fe^{2+} and 1.2×10^{-9} M for Fe^{3+} (Hasegawa and Ichiyama 2005) and this must be taken into account to avoid misinterpretation of the data on the relation between cell proliferation and calcium influx.

4.9 Cyclin, Cyclin-Dependent Kinase, Lipid Rafts, and Calcium Influx

When needed, calcium influx must be strictly regulated to trigger cell proliferation in a well-controlled fashion. Calcium influx can be recorded after stimulation by several different agonists using the phospholipase C and phospholipase A2 pathways in the same cell type. However, it is trivial to say that not all of these agonists are related to cell proliferation. The cell cycle and cell division are under the strict control of cyclins and CDK complexes (Fig. 4.1) (Murray and Hunt 1993). Specific sets are expressed during the different phases of the cell cycle, and it is known that their expression and activity are controlled in some cell types by calcium influx. The expression and activities of cyclins A and E are dependent on SOCE activation. First, SOCE activates calcineurin (Mignen et al. 2003), which in turn controls the activities of cyclins A and E (Tomono et al. 1998). Second, SOCE blockers inhibit induction and activation of cyclins A and E (Tomono et al. 1998; Glassford et al. 2003). Third, the timing of cyclin D activation after serum addition matches the timing of increases in the amplitude of SOCE (Tomono et al. 1998). Calmodulin and calmodulin-dependent protein kinase II are known to play a major role in relaying calcium entry and cell proliferation (Hickie et al. 1983; Means 1994; Takuwa et al. 1995; Colomer and Means 2007). To achieve maximal efficiency and specificity, the plasma membrane must have a high degree of organization, and this can be achieved in specific regions such as lipid raft domains (Brown and London 1998). Early experiments in the 1970s suggested a role of caveolae in regulating calcium entry (Popescu et al. 1974). More recently, it was demonstrated that caveolae are preferred sites for SOCE (Isshiki et al. 2002; Isshiki and Anderson 2003). Interestingly, receptors for two potent mitogens, epidermal growth factors and hepatocyte growth factor, which are known to stimulate TRPC6 expression in human hepatoma cells (El Boustany et al. 2008), are also localized in caveloae (Mineo et al. 1996; Duhon et al. 2010). Other growth factor receptors have been found in caveolae (Pike 2005), and they are known to control and organize calcium-dependent signal transduction (Pani and Singh 2009). Several calcium channels and calcium signaling proteins are found in caveolae (Pani and Singh 2009) and, for example, caveolin has been implicated in TRPC1 targeting the plasma membrane and its association with STIM1 (Brazer et al. 2003; Pani et al. 2009). Caveolin is also involved in ORAI1 internalization during meiosis, hence downregulating the amplitude of SOCE (Yu et al. 2009, 2010). This relation between localization and calcium-dependent signaling transduction has been illustrated by studies showing specific activation of downstream transduction pathways by one particular calcium channel (Dolmetsch et al. 2001;

Wheeler et al. 2008). It is also clear that NFAT activation is tightly linked to I_{CRAC} but not to I_{arc} in HEK293 cells (Mignen et al. 2003).

The nature of the calcium channel involved in cell proliferation is not known and is likely to differ from one cell type to another. In the case of SOCE and NCCE, STIM1 is associated with ORAI1, ORAI3, and with some TRPC channels. Indeed, I_{CRAC} (Hoth and Penner 1992) now appears to be strictly related to ORAI1 (Cahalan et al. 2007), although a recent study suggested that I_{CRAC} is ORAI3-dependent in MCF-7 cells (Motiani et al. 2010). How can these channels activate cyclins and CDK independently of any other cellular effects remains elusive, but particular attention should be paid to potential new functions for calcium channels. A parallel can be made with InsP3 receptors and their modulation by the complex Cdc2, the human homolog of CDK1, and cyclin B in Jurkat cells (Malathi et al. 2005). ORAI and TRPC channels are known to bind to an ever-growing number of cellular proteins, but a link with cyclins and CDK is yet to be established. Indeed, this link could involve downstream events regulating cyclin/CDK complex expression and activity and could also result from an increase in cytosolic calcium concentration independently of activation of calcium entry. However, recent evidence suggesting additional roles for calcium channels is likely to support this strong hypothesis.

4.10 Additional Functions for Calcium Channels

Recent work has demonstrated that many ion channels can directly influence biochemical events in ways that do not directly depend on their function as ion channels (Kaczmarek 2006). As pointed out by Kaczmarek (2006), although many signaling molecules are known to associate with ion channels, the finding that an integral element of the channel also catalyzes an enzymatic reaction or participates in cell–cell interactions strongly suggests that changes in channel activity could influence these processes. New functions for voltage-gated calcium channel auxiliary β subunits have been identified recently. These functions appear to be regulated by the β subunit alone, independently of any effects on the calcium influx; hence, the β subunit may not be truly "auxiliary" and may play more fundamental roles in calcium homeostasis or gene regulation (Rousset et al. 2005; Barbado et al. 2009). More recently, a proteolytic fragment of the C-terminus of the pore-forming subunit of L-type channels (Cav1.2) was shown to act as a transcription factor (Gomez-Ospina et al. 2006). Members of the TRPM subfamily have longer cytoplasmic C-terminal domains than other TRP channels and were the first ion channels shown to have enzymatic activity (Huang 2004). As a result, members of this family were named "chanzymes" (Montell 2003). The C-terminus of TRPM2 encodes an ADP-ribose pyrophosphatase domain that binds ADP-ribose and NAD, both of which directly activate the channel (Sano et al. 2001; Zhang et al. 2006). TRPM6 and TRPM7 both contain protein kinases in their C-terminal domains (Schmitz et al. 2005). These kinases are capable of phosphorylating both the channels themselves and other substrates. It is therefore possible that new functions for TRPC and ORAI channels could be uncovered soon and that a direct link between the channel structure and transcription factors would

explain why some cells need the channel more than calcium influx to proliferate. As a matter of fact, our preliminary results (unpublished data) show that cell proliferation in HEK293 cells strictly depends on ORAI1 and ORAI3 expression and is totally independent of external calcium. The mechanisms by which these channels can control the cell cycle and cell division are still unknown, but it is clear that they can interact with more than a single intracellular protein, and we are currently investigating which region of these channel proteins is essential for this physiological response.

4.11 Endoplasmic Reticulum Calcium Content and Cell Proliferation

We do not exclude that calcium influx may just regulate ER calcium contents and that in fact cell proliferation only depends on the state of ER refilling. Knocking down calcium channels, blocking the cell cycle using different drugs, incubation in serum-free solution, use of calcium blockers, and incubation in calcium-free solution all often result in a decrease in ER calcium contents. It has been known for years that ER calcium contents modulate cell proliferation (Short et al. 1993; Waldron et al. 1994; Magnier-Gaubil et al. 1996; Lipskaia et al. 2009). Increase in ER calcium contents was correlated to an increased rate of cell proliferation in prostate cancer cells and conversely (Legrand et al. 2001). This is indeed linked to changes in expression of SERCA isoforms as well as modulation of expression (Lipskaia et al. 2009). The conflicting data of increased and decreased SERCA expression in cancer may be due to a shift in SERCA expression from one isoform to another rather than a change in overall SERCA expression (Bergner and Huber 2008). The kinetics of expression of ORAI1 channels and SOCE recovery after serum starvation depend on the cell type. A fast and full reversion of both effects was observed in less than 4 h in HEK293 cells (El Boustany et al. 2010), whereas I_{CRAC} recovery took about 24 h in RBL cells (Bodding 2001). However, in our study, ER calcium contents were still low 4 h after readdition of serum, whereas SOCE amplitude was fully recovered in HEK293 cells (unpublished data). Proper kinetic studies on the relation between ER calcium contents and SOCE amplitude during serum starvation and after addition of serum should establish the relative importance of these two sources for calcium ions in cell proliferation.

4.12 Conclusion and Perspectives

To conclude, the relationship between calcium influx and cell proliferation should not be taken for granted in all cell types and in all physiological conditions. Better knowledge of the real nature of the calcium channels involved in each cell type should provide information on how calcium entry controls all the downstream events leading to cell proliferation. Establishing new functions for calcium channels in cell proliferation independently of calcium flux, looking for specific

blockers, and studying calcium influx during the cell cycle in the absence of any kind of chemically induced block are probably the next steps needed to clarify the role of calcium influx in cell proliferation and cell cycle progression.

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Chapter 5
Role of Calcium Signaling in Stem and Cancer Cell Proliferation

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Abstract Calcium (Ca^{2+}) is a ubiquitous second messenger involved in the regulation of many cellular activities. Importantly, both cytosolic and nuclear Ca^{2+} signals have essential roles in the progression through the cell cycle. Ca^{2+} signals in the these subcellular compartments are generated through the concerted action of several components of the Ca^{2+} signaling machinery that reside in the plasma membrane, cytosol, nuclear envelope membrane, or the nucleus. The versatility and specificity of Ca^{2+} signals is determined by their spatial and temporal patterns, and Ca^{2+} signals can be regulated independently of cytosolic Ca^{2+} signals. This review discusses the machinery involved in cytosolic and nuclear Ca^{2+} signal formation, as well as the different mechanisms through which these Ca^{2+} signals modulate the process of cell proliferation.

Keywords Calcium signaling • Stem cells • Cells proliferation • Transcription factors • Cancer • Nucleoplasm • Calcium channels • Cell cycle

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5.1 Introduction

Many cellular functions are regulated by increases in intracellular Ca^{2+} concentration (Berridge et al. 1998). There is increasing evidence that this versatility and specificity of Ca^{2+} signals is determined by their spatial and temporal patterns (Berridge et al. 2003). Ca^{2+} signals can originate from intracellular stores or from the external medium. In the latter case, there are a myriad of plasma membrane channels that control Ca^{2+} entry from the extracellular space in response to several stimuli (see below). Release of Ca^{2+} from intracellular stores is mediated by several intracellular messengers [Ca^{2+} itself, inositol 1,4,5-trisphosphate (IP_3), cyclic ADP ribose (cADPR), and nicotinic acid adenine dinucleotide phosphate (NAADP)], that bind to and regulate the activity of specific intracellular receptors to promote Ca^{2+} release (Chini 2002; Berridge et al. 2003).

In particular, IP_3-mediated Ca^{2+} release is initiated by the binding of Ca^{2+}-mobilizing hormones or growth factors to plasma membrane G-protein-coupled receptors (GPCRs) (Simon et al. 1991; Berridge 1995) or receptor tyrosine kinases (RTKs) (Berridge 1995; Lev et al. 1995), respectively. Once these mobilizing agents bind to their respective receptors, phospholipase C (PLC) is activated and hydrolyzes the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) in the plasma membrane, resulting in the formation of diacylglycerol (DAG) and IP_3. DAG remains at the plasma membrane to activate protein kinase C (PKC), whereas IP_3 diffuses into the cytosol to bind to IP_3 receptors (IP_3Rs), causing receptor opening and subsequent release of Ca^{2+} from intracellular stores (Berridge et al. 2003). As will be discussed, growth factor receptor signaling involves translocation of several RTKs to the nucleus to generate Ca^{2+} signals there (Gomes et al. 2008). In fact, most Ca^{2+} signaling machinery found in the cytoplasm is duplicated in the nucleus, and there is evidence that nuclear Ca^{2+} signals can occur independently of cytosolic Ca^{2+} signals (Echevarria et al. 2003; Leite et al. 2003). Thus, increases in nuclear Ca^{2+} concentration have specific biological effects that differ from the effects of increases in cytoplasmic Ca^{2+} concentration. These effects include the activation of distinct genes and transcription factors (Hardingham et al. 1997; Carrion et al. 1999; Pusl et al. 2002), activation of intranuclear kinases (Deisseroth et al. 1998; Echevarria et al. 2003), and regulation of cell proliferation (Rodrigues et al. 2007; Andrade et al. 2011; Smyth et al. 2010). Release of Ca^{2+} from intracellular Ca^{2+} stores is followed by stimulation of the plasma membrane Ca^{2+}-release-activated Ca^{2+} (CRAC) channel (Liou et al. 2005; Roos et al. 2005; Zhang et al. 2006), which involves the pore-forming units Orai1, Orai2, and/or Orai3 (Prakriya and Lewis 2001; Ma et al. 2002). The Orai isoforms are activated by the Ca^{2+}-sensing proteins stromal interaction molecule (STIM) 1 and STIM2, which are activated on depletion of Ca^{2+} from the endoplasmic reticulum (ER) (Manji et al. 2000; Williams et al. 2001). The stimulation of Orai isoforms by STIM is followed by activation of the CRAC current (I_{CRAC}) and store-operated Ca^{2+} entry (SOCE), which is an alternative mechanism that accounts for sustained calcium entry and regulates cell proliferation (Kurosaki and Baba 2010).

SOCE is critical for several physiological functions, including activation of immune cells and skeletal muscle development (Feske 2009). Following agonist stimulation of nonspontaneously depolarizing cells (e.g., T cells, mesenchymal stem cells, cancer stem cells, hepatocytes, and probably others), Ca^{2+} influx through SOCE allows these cells to proliferate by initiating reentry into the cell cycle (Capiod 2011).

By using an RNA interference (RNAi)-based screen to identify genes that alter thapsigargin-dependent Ca^{2+} entry (Roos et al. 2005) and an experimental system using *Drosophila* S2 cells in which expression of several targeted gene products was individually suppressed by RNAi and evaluated for their role in store-operated channel (SOC) influx, (Roos et al. 2005) two independent groups identified STIM1 as the ER Ca^{2+} sensor. One year later, with use of a genome-wide RNAi screen (Feske et al. 2006; Vig et al. 2006; Zhang et al. 2006) together with previous information in which cells from patients are defective in SOCE and CRAC channel function (Feske et al. 2005), Orai1 was found as the SOCE channel at the cell membrane. The protein STIM1 is a regulator of the store-operated Ca^{2+} influx and CRAC function. Thus, a single EF-hand Ca^{2+}-binding motif located near the intraluminal N terminus of STIM1 may sense the decrease in free Ca^{2+} stores (Liou et al. 2005; Zhang et al. 2005). Indeed, how this information is transmitted to the plasma membrane Ca^{2+} entry channels is relevant. When the store was emptied, it was observed that STIM1 undergoes a profound redistribution within the cell (Zhang et al. 2005). Whereas labeled STIM1 normally appears to be uniformly distributed within the ER, store depletion leads to the appearance of densely labeled puncta, indicating that STIM1 becomes reorganized into spatially discrete areas after Ca^{2+} release from stores (Fig. 5.1) (Liou et al. 2005; Stathopulos et al. 2008).

Orai1 is a plasma membrane protein with four predicted transmembrane segments containing a putative coiled-coil motif in the C terminus that forms the channel (Zhang et al. 2006; Muik et al. 2008). Orai proteins share no homology with any other ion channel family of any other known protein. Recent studies have shown that Orai1 forms multimers and that targeted mutations in Orai1 alter the conductance properties of the CRAC channel (Prakriya et al. 2006; Vig et al. 2006; Yeromin et al. 2006; Gwack et al. 2007). Coexpression of STIM1 and Orai1 results in an enormous gain in function of SOCE and CRAC channel activity, indicating that the plasma membrane Orai1 protein is likely the channel entity mediating SOCE, thus replicating the biophysical properties of the CRAC current (I_{CRAC}) (Peinelt et al. 2006; Soboloff et al. 2006; Zhang et al. 2006).

5.2 STIM1 and Orai1 Interaction and Movement on the Cell Membrane

STIM1 and Orai1 are present in a wide variety of cells from different tissues and have a main role in regulating Ca^{2+} signaling (Fig. 5.1) (Cahalan et al. 2007; Frischauf et al. 2008). Orai1 is also critically important for the regulation of cell proliferation (Baryshnikov et al. 2009; Yu et al. 2010; Faouzi et al. 2011b).

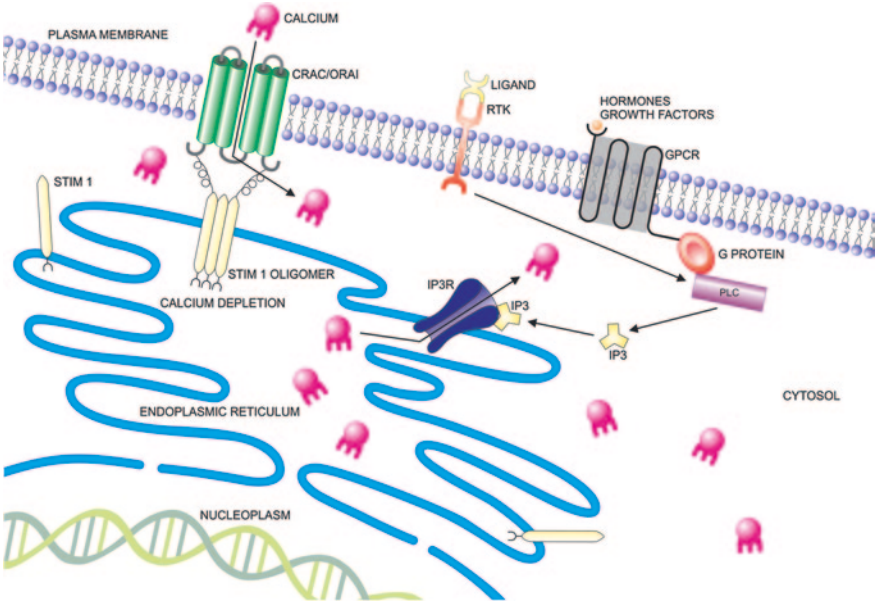

Fig. 5.1 Mechanisms involved in increase of cytosolic Ca^{2+} concentration. Increase of cytosolic Ca^{2+} concentration includes release of Ca^{2+} from intracellular stores entry of Ca^{2+} through the plasma membrane. The activation of plasma membrane receptors, i.e, receptor tyrosine kinases (*RTK*) and G-protein-coupled receptors (*GPCR*) by their ligands promotes a cascade of events resulting in production of inositol 1,4,5-trisphosphate (*IP3*). In the endoplasmic reticulum, IP3 binds to its receptor (*IP3R*), causing the release of stored calcium. The decrease in ER calcium concentration is perceived by stromal interaction molecule 1 (*STIM 1*) receptors, which translocate to the membrane, forming oligomers (puncta). STIM1 binds to the Ca_{2+}-release-activated Ca^{2+} (*CRAC*)/ORAI channels (calcium channels activated by calcium release), promoting the store-operated Ca^{2+} entry. *PLC* phospholipase C

Moreover, SOCE has been shown to participate in cell migration (Wei et al. 2009, 2012). In fact, it has also been implicated in breast tumor metastasis (Yang et al. 2009; Eylenstein et al. 2012).

Given the paramount role of SOCE-dependent Ca^{2+} signaling in the regulation of cell function, Orai1 and STIM1 are expected to be under tight regulation by a wide variety of cellular mechanisms. A recently described signaling pathway related cyclic adenosine 3′,5′-monophosphate (cAMP) generation to the subplasmalemmal accumulation of STIM1 (Tian et al. 2012). It was suggested that cAMP induces STIM1 translocation since dynamin-related mitochondrial protein mitofusin 2, which was recently implicated in the trafficking of STIM1 to the ER–plasma membrane junctions (Singaravelu et al. 2011), is regulated by protein kinase A (PKA) (Zhou et al. 2010). Moreover, mitochondrial uptake of Ca^{2+} entering through CRAC channels reduces Ca^{2+}-dependent slow inactivation of the channels and mitochondrial depolarization impairs movement of STIM1 across the ER membrane (Bakowski et al. 2012). Additional processes are likely involved, as translocation is induced by both specific PKA and Epac agonists.

However, although the STIM1 translocation determined by the filling state of the ER affected STIM1–Orai1 co-clustering and modulated SOCE as expected, translocation determined by cAMP occurred without an effect on Orai1 and Ca^{2+} entry (Tian et al. 2012). Moreover, association between STIM1 and Orai1 is enhanced by Homers. Homer is a family of cytoplasmic adaptor proteins that plays different roles in cell function, including the regulation of CPCRs. These proteins contain an Enabled/vasodilator-stimulated phosphoprotein homology 1 domain that binds to the PPXXF sequence motif, which is present in different Ca^{2+}-handling proteins, such as IP_3Rs and transient receptor potential canonical (TRPC) channels (Jardin et al. 2012).

Another related pathway which is present in apoptosis resistance, and in invasive growth of diverse tumor types and metastasis is promoted by the constitutive activation of protein kinase B (PKB; also known as Akt) (Madhunapantula and Robertson 2009). In normal cells, activation of PKB through its phosphorylation by phosphatidylinositol 3-kinase is counterbalanced by the action of protein phosphatase 2A (PP2A), protein phosphatase 1, and tensin homologue on chromosome 10 lipid phosphatase, which interfere with removal of activating phosphates from the membrane and trafficking of PKB (Westermarck and Hahn 2008; Madhunapantula and Robertson 2009). However, in melanoma cells, those counterbalanced actions do not function properly to maintain this mechanism owing to several genetic disorders such as the loss of phosphatase and tensin homologue on chromosome 10, Ras oncogene mutations, or amplification of RTKs and PKB-isoform-encoding genes (Westermarck and Hahn 2008; Madhunapantula and Robertson 2009; Nazarian et al. 2010). Once deregulated, PKB elicits its tumor-promoting function in cooperation with the oncogene B-Raf (Madhunapantula and Robertson 2009; Paraiso et al. 2011), but importantly overrides the melanoma-suppressive effect of pharmacological B-Raf blockers (Nazarian et al. 2010; Shao and Aplin 2010; Paraiso et al. 2011). These observations highlight the central role of this enzyme in melanoma malignancy. PP2A dysfunction in cancer has been related to, but not only, mutational changes of the regulatory PP2A subunits (Nazarian et al. 2010) and overexpression of the endogenous phosphatase inhibitors (Shao and Aplin 2010). Studies on diverse cancer cell types such as leukemia (Liu et al. 2003), colon (Barisic et al. 2010) and breast (Wong et al. 2009) carcinoma cells demonstrated that the action of PP2A could be repressed through phosphorylation of its catalytic enzyme subunit by activated Src or other nonreceptor tyrosine kinases and RTKs (Westermarck and Hahn 2008; Madhunapantula and Robertson 2009). Furthermore, it was demonstrated in neurons and osteoblastic cell types that the inhibition of PP2A through Src activation and thereby Src-induced phosphorylation with increases in intracellular Ca^{2+} concentration correlated well with the activation of the Src/PP2A/PKB pathway by the Ca^{2+} sensor calmodulin (CaM) in malignant cells (Fig. 5.2), in which accelerated SOC Ca^{2+} influx maintains moderately elevated cytosolic Ca^{2+} concentration (Feldman et al. 2010).

SOCE is a predominant Ca^{2+} entry route in nonexcitable cells, stimulated by many membrane receptors and mediated by depletion of intracellular Ca^{2+} stores. Lipid rafts have been reported to modulate SOCE by creating a platform for interactions of different SOC-associated proteins (Alicia et al. 2008; Fedida-Metula et al. 2008). Particularly, STIM1 targeted to the rafts might assemble with TRPC and/or Orai constituents of the

store-operated Ca^{2+} permeation pore to elicit Ca^{2+} entry. Growing evidence now implicates robust SOC responses in enhanced proliferation, survival, and invasion of melanoma (Feldman et al. 2010), breast (Faouzi et al. 2011a), colon (Wang et al. 2012a), lung (Hou et al. 2011), and hepatocellular (El Boustany et al. 2008) carcinoma cells. However, the pathways linking these channels to sustained deregulation of major tumor-promoting signaling elements are not fully understood. The recruitment of STIM1 to melanoma rafts, triggering Ca^{2+} influx and Ca^{2+}/CaM-dependent stimulation of the Src/PP2A/PKB cascade (Fedida-Metula et al. 2012), demonstrates for the first time that such a link is mediated by cholesterol-enriched microdomains connecting SOC-induced rises in cytosolic Ca^{2+} concentration to high basal PKB activity, which is critical for melanoma malignancy (Fig. 5.2). Until recently the identity of the raft-associated Src activator, which involves the coupling of CaM to Src, was not known. It remains to be determined whether the latter activators are adaptor proteins, such as arrestins (Wu et al. 2006a), or RTKs and GPCRs (Sanchez-Gonzalez 2010). Notably, stimulation of CaM by the SOC also evokes activation of the transcription factors nuclear factor κB

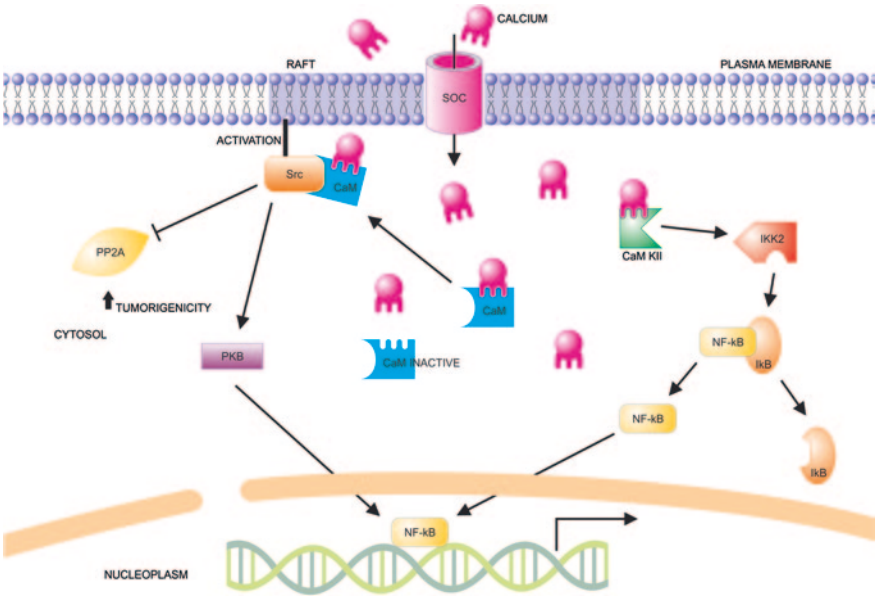

Fig. 5.2 Regulation of the transcription factor nuclear factor κ light chain enhancer of activated B cells (*NF-κB*) by Ca^{2+}. In the cytoplasm, the presence of a Ca^{2+} transient triggers a phosphorylation cascade which culminates in NF-κB activation. Ca^{2+} activates Ca^{2+}/calmodulin (*CaM*)-dependent protein kinase II (*CaM KII*), which promotes activation of IκB kinase (*IKK2*). Once activated, IKK2 phosphorylates and inhibits IκB, which allows IκB to dissociate from NF-κB. Then, NF-κB translocates to the nucleus, where it activates the transcription of certain gene networks, which are generally related to inflammatory or immune responses, cell survival responses, or cell proliferation. In the nucleus, NF-κB can be activated by factors originating in other signaling pathways. For example, activation of Src anchored in lipid rafts in the plasma membrane by CaM leads to blockage of protein phosphatase 2A (*PP2A*), whereas protein kinase B (*PKB*) is activated. The regulation of NF-κB through the activation of PKB is associated with malignant melanoma. *SOC* store-operated channel

(NF-κB) and nuclear factor of activated T cells (NFAT) (Figs. 5.2, 5.3) (Hogan et al. 2003). Recent studies in melanoma (Flockhart et al. 2009), colon (Wang et al. 2012a) and lung (Huang et al. 2011) carcinoma cells indicated a role of these proteins in transcriptional upregulation of metastasis-related genes. Since PKB promotes nuclear translocation and thereby activates both NFAT and NF-κB(Romashkova and Makarov 1999), it would be of particular interest to examine the potential cross talk between PKB and NFAT/NF-κB SOC-driven signaling and its significance for cancer invasion. Regardless of these unresolved issues, the obvious dependence on the Src/PP2A/PKB cascade for lipid raft integrity by itself implies important therapeutic implications, as targeting rafts in vivo might restrain melanoma development by reactivating PP2A and inhibiting PKB at the tumor site (Fig. 5.2) (Fedida-Metula et al. 2012).

Subcellular localization of SOCE channel components may depend on the cell cycle's phase: STIM1, but not STIM2, is expressed at the cell surface during mitosis, whereas it localizes to the ER during interphase (Ercan et al. 2012). Efficient retention of STIM1 in the ER during interphase depends on its lysine-rich domain and a diarginine ER retention signal. Presumably hyperphosphorylation of STIM1 leads to the inactivation of SOCE during cell division, which occurs during both meiosis and mitosis (Smyth and Putney 2012).

Although STIM proteins function both as Ca^{2+} sensors and activators of CRAC channels, the relative roles of STIM1 and STIM2 in supporting physiological Ca^{2+} signals and downstream Ca^{2+}-dependent responses are unresolved. There are differences between STIM proteins. For instance, their abilities to activate Orai1 are different. STIM2 has approximately twofold lower affinity for ER Ca^{2+} levels than STIM1, even for similar levels of Orai1 expression. Thus, a smaller drop in luminal Ca^{2+} concentration is required for STIM2 activation (Brandman et al. 2007). This lower affinity of STIM2 results in its preferential recruitment after modest store depletion (Bird et al. 2009). This difference may be due to the N-terminal domain of the STIM proteins (Zhou et al. 2009), and also to the inhibition effects of cytoplasmic CaM on STIM2–Orai1 coupling (Parvez et al. 2008). At this point, we can speculate that those cells which proliferate at low extracellular Ca^{2+} concentration, as discussed above, could express and present more functional STIM2 than STIM1 at the plasma membrane. However, the definite function of STIM2 is not completely understood. STIM2 has been proposed to be at the center of a feedback module that keeps basal cytosolic and ER Ca^{2+} concentrations within tight limits, in the absence of stimulation, and activated Ca^{2+} influx on smaller decreases in ER Ca^{2+} concentration (Brandman et al. 2007; Graham et al. 2011), in contrast to STIM1, which triggers Ca^{2+} influx in response to receptor-mediated depletion of ER Ca^{2+} stores. STIM2, like STIM1, causes Ca^{2+} influx via activation of the plasma membrane Ca^{2+} channel Orai1 (Bird et al. 2009). Moreover, STIM2 was shown to have two distinct modes for activating CRAC channels: a store-operated mode that is activated through depletion of ER Ca^{2+} stores by IP_3 and store-independent activation that is mediated by cell dialysis during whole-cell perfusion. Both modes are regulated by CaM. The store-operated mode is transient in intact cells, possibly reflecting recruitment of CaM, whereas loss of CaM in perfused cells accounts for the persistence of the store-independent mode. The inhibition by CaM can be reversed by

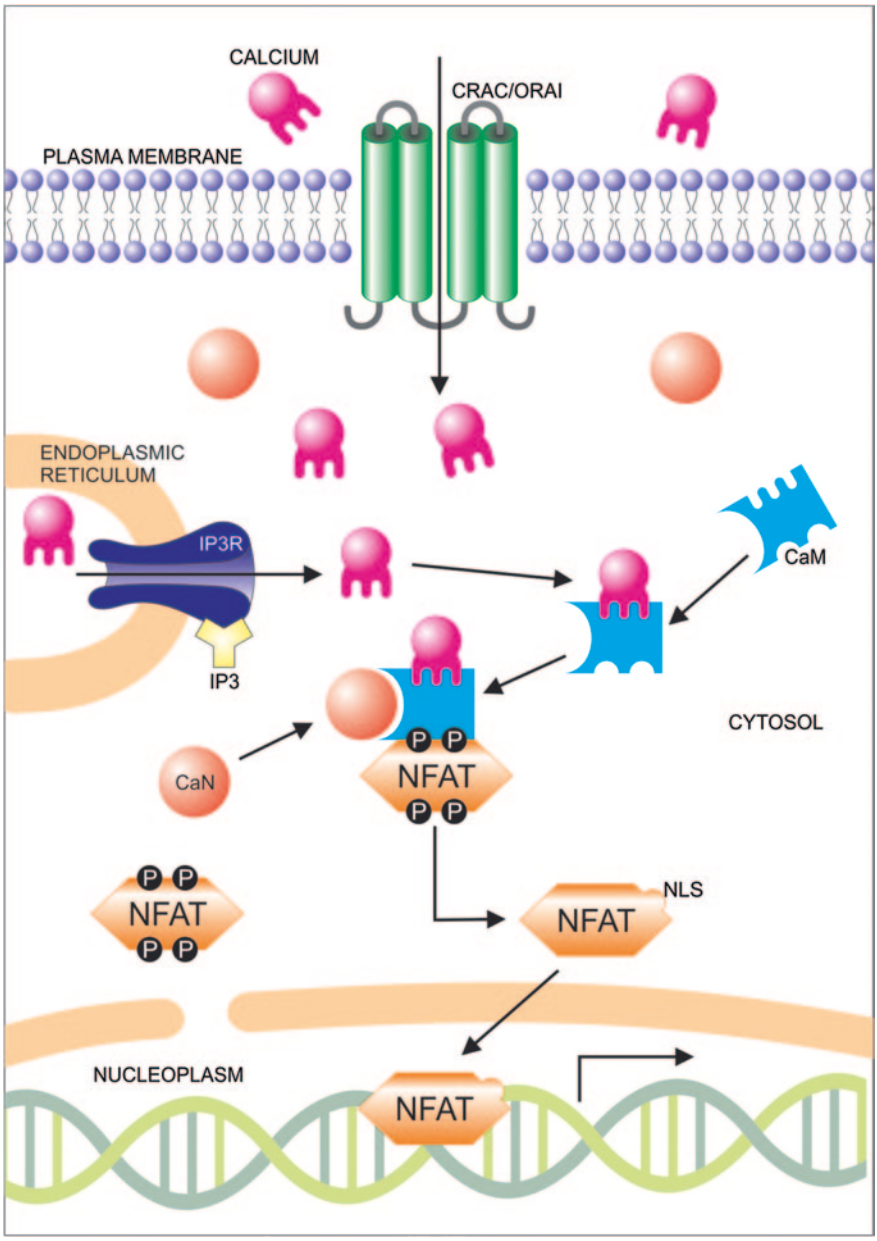

Fig. 5.3 Regulation of the transcription factor nuclear factor of activated T cells) (*NFAT*) by Ca^{2+}. The increase in intracellular Ca^{2+} concentration by IP_3R activation and the influx through CRAC/ORAI channels activates the protein CaM, which binds to and activates the phosphatase calcineurin (*CaN*). Activated CaN dephosphorylates several serine residues in the NFAT proteins, on which a nuclear localization signal (*NLS*) is then exposed. NFAT is then translocated to the nucleus, where it binds to DNA and regulates target genes

2-aminoethoxydiphenyl borate, resulting in rapid, store-independent activation of CRAC channels (Parvez et al. 2008). STIM2 knockdown, but not STIM1 knockdown, significantly lowered basal cytosolic Ca^{2+} concentrations in HeLa cells, human umbilical vein endothelial cells, and human embryonic kidney (HEK) 293T cells (Brandman et al. 2007). This is indeed consistent with STIM2 mediating the persistent signaling of SOCs at a low level of ER Ca^{2+} depletion. Also in HEK293 cells, knockdown of STIM2 had no significant effect either on store Ca^{2+} content or on the frequency of Ca^{2+} oscillations in response to GPCR stimulation. However, in STIM1-deficient cells, Ca^{2+} oscillations fell rapidly and disappeared with a time course similar to that seen on stimulation in Ca^{2+}-free solution (Bird et al. 2009). These results confirmed that STIM1 and not STIM2 supported physiological levels of Ca^{2+} signaling in response to GPCRs.

Despite weak effects on the Ca^{2+} signal (Oh-Hora et al. 2008), STIM2 was nevertheless important for sustaining CRAC-channel-dependent gene expression, measured several hours after stimulation. In T cells, deficiency of either STIM1 or STIM2 impaired sustained Ca^{2+} influx, nuclear translocation of NFAT, and cytokine production. However, mice lacking both STIM1 and STIM2 in T cells developed a lymphoproliferative syndrome characterized by splenomegaly, lymphadenopathy, infiltration of leukocytes into many organs, and a selective decrease in the number and function of regulatory T cells (T_{reg} cells) expressing the lineage-specific transcription factor Foxp3. Similarly, B-cell-specific deletion of STIM1 and STIM2 in mice caused a profound defect in B-cell-receptor-induced SOC influx and proliferation. However, B cell development and antibody responses were unaffected, indicating a possible mechanism regarding cell proliferative pathways (Feske et al. 2012).

Calcium channels and intracellular Ca^{2+} stores were previously demonstrated to regulate diverse cell phenotypes during proliferation (Resende et al. 2008a, b, c, 2010a; Resende and Adhikari 2009; Andrade et al. 2011; Guerra et al. 2011; Ding et al. 2012; Marques et al. 2012; Peters et al. 2012). B-cell-specific deletion of STIM2 revealed that this protein contributed significantly to NFAT-dependent interleukin-10 synthesis, despite only a modest reduction in store-operated Ca^{2+} influx (Matsumoto et al. 2011). Although most of the data have been obtained in immune cells, their intrinsic Ca^{2+}/NFAT pathway which regulates cell proliferation remains similar in diverse blast cells (Holmes et al. 2010; Guo et al. 2011).

One example showing this Ca^{2+}/NFAT pathway in T_{reg} cells is that on CD3 cross-linking, Foxp3 was capable of activating store-operated Ca^{2+} influx, indicating that PLCγ activation, generation of IP_3, store-operated Ca^{2+} influx, and nuclear translocation of NFAT were entirely functional in this cell type. NFAT activation is essential for proper proliferation, development, and function of thymus-derived T_{reg} cells (Wu et al. 2006c, Marson et al. 2007). The defect (deletion of STIM1 and STIM2) in T_{reg} cell development noted in those studies could also reflect the reported "cooperation" between Smad3 and NFAT at an enhancer element in the Foxp3 locus (Tone et al. 2008; Tone and Greene 2011). Further studies are needed to elucidate the complex effects of mutations in the Ca^{2+}/calcineurin/NFAT signaling pathway on the development of conventional T cells and T_{reg} cells.

Kar et al. (2012) demonstrated that different agonists sustain cytoplasmic Ca^{2+} signals and gene expression through activation of different STIM proteins. Stimulation of cysLT1 receptors, a GPCR family member, which evokes large all-or-none baseline Ca^{2+} spikes, supports both repetitive Ca^{2+} oscillations and NFAT-driven gene expression through recruitment of STIM1 to open CRAC channels. Otherwise, STIM2 knockdown had no effect on either Ca^{2+} oscillations or Ca^{2+}-dependent gene expression. Hence, after activation of GPCRs of the $G_{q/11}$ type (Gwozdz et al. 2012), STIM1 is necessary and sufficient to support physiological Ca^{2+} signals and initiate a functional response. It was also demonstrated that STIM1 is phosphorylated during mitosis, failing to rearrange into near-plasma-membrane puncta in mitotic cells, a critical step in the SOCE-activation pathway (Smyth et al. 2009). Interestingly, during development, neural stem cells display spontaneous calcium oscillations generated at the G1–S transition point through activation of two different types of ER receptors, IP_3R and the ryanodine receptor (RyR) (Resende et al. 2010a, b). Recently, a connection between the frequency of spontaneous calcium oscillations, the activity of cell cycle mediators, and the duration of the G1 phase in neural stem cells has been reported. Application of calcium imaging techniques confirmed an increased calcium oscillatory frequency that correlates with downregulation of the cell cycle inhibitor p27kip1, and augmented proliferative potential, and, in accordance with the report of Salomoni and Calegari (2010), a decrease in the duration of the G1 phase (Resende et al. 2010a). Although no direct relationship was demonstrated between calcium oscillations and STIM1 in these reports, others showed a link between cell proliferation/apoptosis, PLC, NF-κB, STIM1, Orai1, and calcium oscillations (Eylenstein et al. 2012; Gomez-Fernandez et al. 2012; Martin-Romero et al. 2012; Parys et al. 2012; Ramadan et al. 2012; Yue et al. 2012). These authors have suggested that the primary function of large Ca^{2+} oscillatory signals might be to ensure sufficient store depletion to activate CRAC channels robustly through STIM1, leading to activation of downstream effectors. In agreement with this view, it was shown that c-fos and NFAT, two different transcription factors, promote local Ca^{2+} concentration elevations near open CRAC channels and activate gene expression (Di Capite et al. 2009; Kar et al. 2011). Thus, we suggest that the role of oscillations might be to activate CRAC channels, thereby ensuring the generation of spatially restricted physiological Ca^{2+} signals driving gene activation, and that alterations in the cell differentiation could be encoded within different ranges of calcium oscillatory frequencies (Tonelli et al. 2012). It was also demonstrated that local Ca^{2+} influx signals to the nucleus much more effectively than a robust bulk Ca^{2+} concentration rise. This leads to the expression of the transcription factor c-fos, a regulator of proinflammatory responses and a critical factor that governs estrogen- and androgen-dependent gene expression and cancer proliferation programs (Dahlman-Wright et al. 2012; Sen et al. 2012). Furthermore, the nonreceptor tyrosine kinase Syk clusters at the cell periphery and couples Ca^{2+} microdomains to c-fos expression through recruitment of the cytoplasmic transcription factor STAT5 in a PKC- and MEK/extracellular-signal-regulated kinase (ERK)-independent pathway (Ng et al. 2009). Parallel processing of the Ca^{2+} microdomain by Syk through two distinct signaling pathways constitutes a novel mechanism to evoke spatially and temporally different cellular responses. In the same

way, androgen binds androgen receptors (ARs) at or near the plasma membrane, which transactivates the epidermal growth factor receptor (EGFR) via matrix metalloproteinase mediated release of EGFR ligands. EGFR can also be activated directly by epidermal growth factor. This activates Src, which phosphorylates PXN on tyrosine residues, enabling Raf to activate MEK/ERK. ERK then mediates phosphorylation of PXN at serine residues, and PS-PXN enters the nucleus. Meanwhile, androgen-bound AR also translocates to the nucleus. In the nucleus, PS-PXN interacts with androgen-bound AR to retain it in the nucleus. PS-PXN then associates with or near the AR on the PSA/NKX3-1 promoter to help promote AR-driven transcription. Activated ERK also enters the nucleus, where PS-PXN, p-ERK, and ELK1 form a complex and ERK phosphorylates ELK1, which induces c-fos expression. The latter then activates cyclin D1 promoter activity/cyclin D1 expression, thereby promoting cell proliferation (Sen et al. 2012).

In contrast to GPCRs, oscillatory Ca^{2+} signals and NFAT-driven gene expression in response to FCεRI receptor activation require both STIM1 and STIM2. Knockdown of either protein leads to a reduced amplitude of the Ca^{2+} spikes as well as a decrease in the number of cells expressing an NFAT-dependent reporter gene. Thus, this raises the question of two different agonists which stimulate the PLC pathway to generate Ca^{2+} oscillations and open CRAC channels activate different combinations of STIM proteins to sustain CRAC channel activity. Ca^{2+} oscillations evoked by diverse GPCR activation exhibit a global amplitude of approximately 1 μM. With a cytoplasmic Ca^{2+} binding capacity of approximately 100 (a calcium binding capacity of 100, for example, means that of 100 calcium ions added to the cytosol, only one ion will remain free and the others will become bound to buffers) (Neher 1995) and with the ER occupying approximately 10 % of the cytoplasmic volume, 1.25 mmol/L Ca^{2+} would have to be mobilized from the ER to generate a typical Ca^{2+} oscillation. As the total concentration of Ca^{2+} stored within the ER is estimated to be 2–5 mmol/L, approximately one quarter to one half of the total available Ca^{2+} pool within the organelle would be mobilized during each Ca^{2+} spike. Each Ca^{2+} oscillation evoked by GPCR agonists would therefore cause a substantial, albeit transient, drop in ER Ca^{2+} concentration, which would exceed the threshold required for STIM1 activation. Thus, Ca^{2+} oscillations in response to cysLT1 receptor stimulation in T_{reg} cells and the subsequent opening of CRAC channels, leading to local Ca^{2+} entry to the nucleus that signals to activate gene expression, depend on STIM1 and not STIM2 (Bird et al. 2009; Kar et al. 2012). An interesting work in which calcium spike amplitude was found to have different roles depending on whether STIM1 or STIM2 is activated demonstrated that unlike GPCRs that activate PLCβ rapidly through a heterotrimeric G protein and, therefore, produce a precipitous but transient drop in ER Ca^{2+} concentration, FCεRI receptor stimulation leads to slower buildup of IP_3 through tyrosine kinase dependent activation of PLCγ (Kar et al. 2012). In the case of IgE, a steady state will therefore arise when the store content has been partially reduced, reflecting a balance between IP_3 production and breakdown. In this way, more STIM1 than STIM2 proteins would probably be activated with this stimulus (Oh-Hora et al. 2008; Kar et al. 2012). However, the question remains

why STIM2 contributes to IgE-evoked Ca^{2+} signals and gene expression but not to those elicited by cysLT1 receptors. It is unlikely that STIM2 is required for STIM1 trafficking because knockdown of STIM2 does not reduce the response to cysLT1 receptor activation. STIM2 gates Orai1 efficiently, because loss of STIM2 had a significant effect on the IgE-evoked Ca^{2+} signal. One possibility is that a signal derived from activated cysLT1 receptors, but not FCεRI receptors, destabilizes STIM2–Orai1 interaction. Alternatively, Parvez et al. (2008) have reported that STIM2 is prevented from activating Orai1 by CaM. It is also possible that the larger Ca^{2+} release generated by cysLT1 receptors might stabilize this inhibitory interaction, whereas the smaller Ca^{2+} release signal to IgE results in a weaker interaction (Kar et al. 2012). Resolution of this issue requires further work.

STIM2 plays a housekeeping role, ensuring that stores are replete with Ca^{2+} before stimulation and contributes to Ca^{2+} signals in response to low levels of receptor activation that deplete stores modestly (Brandman et al. 2007). Stronger levels of stimulation, which would cause a larger cytoplasmic Ca^{2+} concentration rise, switch Orai1 gating to a STIM1-based mechanism.

Finally, any physiopathological condition resulting in a decreased ER Ca^{2+} content could activate constitutive SOCE, unless compensatory mechanisms are present to counterbalance this Ca^{2+} depletion (Sammels et al. 2010). Evidence for ER Ca^{2+} depletion during the cell cycle is sparse. CRAC inhibition has been shown to induce suppression of tumor cell proliferation by arresting the cell cycle at the G0/G1 phase and inhibiting tumor cell metastasis in leukemic cells (Holmuhamedov et al. 2002), breast cancer cells (Yang et al. 2009), and prostate cancer cells (Vanden Abeele et al. 2003). STIM and Orai1 proteins have also been shown to regulate noncancer cells as endothelial cell proliferation (Freichel et al. 2001; Abdullaev et al. 2008). The silencing of Orai1 in human umbilical vein endothelial cells in culture leads to an increase in the proportion of cells in the S and G2–M phases. In the same direction STIM1 or 2 knockdown had a similar but much smaller effect, suggesting that Orai1 might act, at least in part, independently of STIM proteins (Abdullaev et al. 2008). Therefore, the effects of STIM1 knockdown appear to have a cell-type specificity with regard to cellular proliferation.

As stated before, GPCR -induced Ca^{2+} oscillations have unusual characteristics which depend on the type of agonist that activates each receptor. In this case, oscillations may occur over a wide range of agonist concentrations (each receptor has its own range of agonist activation), and the oscillation frequency does not appear to depend on the level of stimulation; they are also inhibited by PKC inhibitors. It is thus plausible that depending on the receptor type, different oscillatory mechanisms would prevail. It was also demonstrated using mathematical models that both IP_3R-based and IP_3-metabolism-based oscillatory mechanisms are considered at the same time (Kummer et al. 2000; Hofer et al. 2002; Resende et al. 2008c; Resende and Adhikari 2009; Aguiar et al. 2010; Guerra et al. 2011; Arantes et al. 2012). This mechanism should allow the rate of IP_3 synthesis to control the period of IP_3R-based Ca^{2+} oscillations through the regulation of either PLC or PKC by Ca^{2+}.

The frequency and amplitude of Ca^{2+} spikes depend on the underlying oscillatory mechanism (Song et al. 2012). Considering the IP_3 metabolism model as the basis of Ca^{2+} oscillations, a sudden increase in IP_3 concentration will disrupt the Ca^{2+} oscillation pattern, causing a delay in the generation of the next Ca^{2+} spike. This delay may correspond to the time that is necessary to restore the IP_3 concentration to its normal range during oscillatory cycles. Otherwise, Ca^{2+} oscillations can occur with a constant level of IP_3, in which there are successive cycles of activation/inhibition of the IP_3R. In this case, oscillations in IP_3 levels lead to local increases in Ca^{2+} levels, which may then propagate as waves in the cytoplasm. This transition may be due to the fact that the rise in IP_3 levels leads to an increase in the number of channels participating in the Ca^{2+} dynamics. In this manner, the effect of fluctuations becomes rather small, because of the low number of clusters, allowing the transition to a deterministic regime (a regime in which the behavior of the system can be predicted) as opposed to a stochastic regime (characterized by random events). Thus, the variability decreases when the frequency increases (Dupont et al. 2008; Dupont and Combettes 2009).

The IP_3Rs in clusters are separated from each other by a few micrometers, suggesting that Ca^{2+} dynamics are intrinsically stochastic, even at the cellular level. As Ca^{2+} is a poorly diffusible messenger in the cytoplasm, the rise in Ca^{2+} concentration occurring at one puff site would be unable to activate release from adjacent sites, which would prevent global signaling. In this manner, Ca^{2+} waves require a sufficient number of IP_3R clusters to become active at the same time in order for them to occur. This process, called nucleation, would lead to a Ca^{2+} concentration increase that is large enough to activate all the IP_3-bound IP_3Rs and generate a Ca^{2+} spike (Dupont et al. 2011).

The Ca^{2+} oscillation patterns differ considerably among cell types and specific cell functions. The release of Ca^{2+} from intracellular stores is generally accompanied by an increased influx of Ca^{2+} across the plasma membrane, which may be induced by either maximal or submaximal concentrations of Ca^{2+}-mobilizing agonists (Putney et al. 1981). Ca^{2+} oscillations are decreased in the absence of extracellular Ca^{2+}, suggesting a requirement for Ca^{2+} influx for their maintenance. However, at least in some nonexcitable cells, Ca^{2+} influx does not appear to be required to drive the oscillations (Bird and Putney 2005), but relies on SOCE (Putney and Bird 2008).

5.3 Structure of the Nuclear Envelope

A brief history of the structure of the nuclear envelope (NE) begins in the early 1990s, when Terasaki and Jaffe (1991) identified the ER as a single membrane system and reported that some molecules can rapidly diffuse within the luminal space defined by the ER and the NE membranes (Subramanian and Meyer 1997). This continuous space has different specialized subdomains: the rough ER, which is studded with ribosomes, and is typically involved in coupling protein synthesis

to protein translocation into the ER lumen through the translocon; the transitional ER sites, which are specialized cup-shaped ER cisternae from which newly synthesized proteins exit via COPII vesicles on route to the remainder of the secretory pathway; and the smooth ER, which is dedicated to calcium storage and metabolic pathways involved in drug handling and lipid and steroid synthesis (Voeltz et al. 2002; English et al. 2009; Pendin et al. 2011). These subdomains are functionally and morphologically distinct, and even though some proteins are characteristic of a particular domain (such as those involved in translocation or processing of newly synthesized proteins, which are enriched in the rough ER), others are shared among them (reviewed in Levine and Rabouille 2005). The NE possesses cell-type-specific differences in the composition of protein subcomplexes, which suggests a functional complexity (Schirmer and Gerace 2005). The NE separates the nuclear and the cytoplasmic compartments of interphase cells and is composed of a double membrane layer and can be subdivided into three morphologically and biochemically different structures: the outer nuclear membrane (ONM), the inner nuclear membrane (INM), and the nuclear pore complex (NPC) (Gerace and Burke 1988; Baumann and Walz 2001). Only the ONM is continuous with the ER, and it is decorated with ribosomes and sharing proteins. However, the ONM also displays a set of unique proteins that can tether the nucleus to the actin cytoskeleton and to the centrosome, and some interact through the lumen with proteins of the INM. The INM contains many distinct proteins that contact the underlying lamina and chromatin (Prunuske et al. 2006; Batrakou et al. 2009; Wilkie et al. 2011). The ONM and the INM are continuous at each NPC that spans the NE and regulate the selective exchange of proteins between the nucleoplasm and the cytoplasm. Electron microscopy and conventional microscopy (Bourgeois et al. 1979) allowed the identification of invaginations or infoldings in the NE of many cell types (Clubb and Locke 1998; Wittmann et al. 2009; Langevin et al. 2010).

NE invaginations are classified as types I and II depending on whether the ONM is involved (Malhas et al. 2011). Type I invaginations are those in which the INM invaginates into the nucleoplasm, whereas type II invaginations involve both the INM and the ONM (Schermelleh et al. 2008). In particular, type II invaginations contain tubules which in turn contain cytoplasmic components such as actin (Clubb and Locke 1998; Johnson et al. 2003) and mitochondria (Clubb and Locke 1998; Lui et al. 2003). These invaginations, which were termed the nucleoplasmic reticulum (Echevarria et al. 2003), increase the area of exchange between the cytoplasm and the nucleoplasm, making contact deep inside the nucleus with chromatin, and sometimes extend to near the nucleolus. Invaginations also increase the number of NPCs providing anchoring sites for active genes and enhancing their expression (Akhtar and Gasser 2007), modify nuclear geometry (which is relevant for diffusion in and out of the nucleus), and form unequally sized nuclear compartments. Indeed, the division of the nucleus into unequally sized compartments by infolding can function as microdomains generating distinct Ca^{2+} signals (Wittmann et al. 2009). Nuclear infolding decreases the diffusion distances. Therefore, Ca^{2+} reaches central sites faster and is cleared more quickly, thus reducing "nuclear inertia" (Queisser et al. 2011). Studies in hippocampal neurons

show that when cytosolic Ca^{2+} transients induce nuclear Ca^{2+} transients, the latter are larger in small nuclear compartments than in large compartments of the same nucleus. Moreover, information encoded in the Ca^{2+} oscillation frequency is preserved and resolved better in small compartments of infolded nuclei. Furthermore, the presence of nuclear infoldings is closely related to gene transcription events, since phosphorylation of histone H3 during synaptic activity was greater in infolded nuclei of neurons than in near-spherical nuclei (Wittmann et al. 2009). This work highlights the translation of synaptic-activity-induced signaling events into changes in nuclear geometry facilitating the relay of Ca^{2+} signals to the nucleus, which may lead to the formation of nuclear signaling microdomains, and could enhance signal-regulated transcription.

The presence of NE invaginations depends on the physiological status of the cell and on the mechanical environment (e.g., stretching of fibroblasts induces loss of nuclear invaginations (Langevin et al. 2010). Furthermore, it was demonstrated that cells that are highly dedifferentiated or cancerous have an increased incidence of invaginations (Johnson et al. 2003).

There are differences between the NE and the peripheral ER; the NE is often described as a subdomain of the ER and the peripheral ER is described as an extensive network branching out of the NE (Mauger 2012). The ONM and the peripheral ER both have similar protein composition, and are involved in Ca^{2+} signaling. The ER is a network that expands within the cytosol and covers every part of the cell and is stabilized partly through interactions with cellular organelles such as mitochondria, the Golgi apparatus, and endosomes or the plasma membrane. These close juxtapositions of membranes are important for interorganelle exchanges and Ca^{2+} signaling. Protein complexes allow mitochondria–ER attachment and transfer of Ca^{2+} to the mitochondria (de Brito and Scorrano 2010). As mentioned above, the ER STIM proteins sense a decrease in the ER Ca^{2+} concentration and activate the plasma membrane ORAI Ca^{2+} channel across the junction between the two membranes, controlling local Ca^{2+} flux (Courjaret and Machaca 2012). The NE has additional roles regulating the transfer of molecules, including Ca^{2+}, from the cytoplasm to the nucleus, and it acts as a Ca^{2+} store that allows delivery of Ca^{2+} to the nucleoplasm (Mauger 2012).

5.4 The NE as a Ca^{2+} Store: Role of the NE in Nuclear Ca^{2+} Signaling

The NE not only isolates the nucleoplasm from the cytoplasm, but also has the ability to store and release Ca^{2+} (Nicotera et al. 1989; Lanini et al. 1992). This can occur through the action of different second messengers such as IP_3 (Nicotera et al. 1989; Gerasimenko et al. 1995), cADPR (Gerasimenko et al. 1995; Adebanjo et al. 1999), and NAADP (Gerasimenko et al. 2003). Sarcoplasmic reticulum/ER Ca^{2+}-ATPase (SERCA) uptake pumps are found in the ONM and have been shown to be identical to those in the ER (Lanini et al. 1992). Additionally, in

some cell types, a Na^+/Ca^{2+} exchanger present in the INM may transfer Ca^{2+} from the nucleoplasm to the lumen of the NE (Xie et al. 2002; Wu et al. 2009). Furthermore, both functional IP_3Rs and RyRs are localized in the nucleoplasmic reticulum (Leite et al. 2003; Marius et al. 2006). IP_3Rs can be found in both the INM and the ONM (Mak and Foskett 1994; Stehno-Bittel et al. 1995; Humbert et al. 1996), and RyRs seem to be present on both leaflets of the NE (Humbert et al. 1996). In particular, IP_3Rs have been found in the nucleoplasmic reticulum of HeLa cells (Lui et al. 2003), liver cells (Echevarria et al. 2003), MDCK cells (Collado-Hilly et al. 2010), smooth muscle cells (Avedanian et al. 2011), and cardiomyocytes (Guatimosim et al. 2008). Moreover, RyRs have been visualized in the NE of nuclei isolated from pancreatic cells (Gerasimenko et al. 2003), in the NE of cardiac cells (Abrenica and Gilchrist 2000), and in the NE and the nucleoplasmic reticulum of myoblasts (Marius et al. 2006). Therefore, the nucleus possesses the machinery necessary for local Ca^{2+} release and reuptake. Such machinery may be activated selectively through tyrosine kinase pathways, since activation of the hepatocyte growth factor (HGF) receptor c-Met or the insulin receptor causes their rapid translocation to the nucleus, in order to selectively hydrolyze nuclear PIP_2 and generate IP_3-dependent nuclear Ca^{2+} signals (Gomes et al. 2008; Rodrigues et al. 2008).

Indeed, the localization of Ca^{2+}-releasing channels is critical for the generation of spatially complex signals and to induce nuclear Ca^{2+} signals (Berridge et al. 2000). Thus, the nucleoplasmic reticulum is a specialized cellular compartment and an additional regulatory Ca^{2+} domain within the nucleus (Malhas et al. 2011), which is involved in the spatial and temporal control of specific intracellular signaling events.

Importantly, nuclear Ca^{2+} signaling may also occur by passive transmission of cytosolic Ca^{2+} signals into the nucleus through the NPCs. In fact, this was originally thought to be the only and principal mechanism responsible for formation of nuclear Ca^{2+} signals (Alonso and García-Sancho 2011). Certainly, even though Ca^{2+} signals can originate in the interior of the nucleus, there is evidence that nuclear Ca^{2+} signals can passively follow cytosolic Ca^{2+} signals (Lin et al. 1994; Fox et al. 1997).

Molecules larger than 70 kDa are considered not to redistribute themselves passively between the nucleus and the cytoplasm. A target sequence is necessary for their transport through the NPC either by facilitated diffusion or by active transport. However, species up to 10 kDa, such as Ca^{2+}, can freely cross through the NPC (Torok 2007). It has been suggested that Ca^{2+} ions and hormones that increase cytosolic Ca^{2+} concentration also play a role in regulating the permeability of the NPC (O'Brien et al. 2007). Also, one of the regulatory roles of NE Ca^{2+} is to change the conformation of the NPC to control the diffusion of intermediate-sized molecules (Strasser et al. 2012). This may provide a mechanism to mediate the entry of transcription factors or other regulatory molecules and to regulate gene transcription in target cells (Sarma and Yang 2011). However, there are some aspects that must be discussed: (1) this does not imply that Ca^{2+} is uniformly transported, (2) it is controversial whether gene transcription can be regulated by

NE Ca^{2+} (Gerasimenko and Gerasimenko 2004), and (3) Ca^{2+}-sensitive indicator and cellular autofluorescence intensities can give rise to artifacts and apparent differences between cytosolic and nuclear Ca^{2+} (Takahashi et al. 1999). These data clearly indicate that the NE of many cell types has all the characteristics of a Ca^{2+} store which can be mobilized on stimulation to deliver Ca^{2+} signals within or in close proximity to the nucleus. A list of key proteins, lipids, and carbohydrates that are present in the nucleoplasmic membrane is provided in Table 5.1.

5.5 Production of IP_3 in the Nucleus

Mobilization of intracellular Ca^{2+} involves the activation of either GPCRs or RTKs, which induces the hydrolysis of PIP_2 and the production of IP_3 (Parys and De Smedt 2012). Many of the various components of the Ca^{2+} signaling cascade have also been found in the nucleus and in parts of the nucleoplasmic reticulum (Table 5.1). Phosphoinositides and the enzymes responsible for their synthesis (phosphatidylinositol and phosphatidylinositol 4-phosphate kinases) are well known to be present in the nucleus (Irvine 2003). Moreover, different PLC isoforms have been identified in the nucleus and may allow local production of IP_3 and DAG (Cocco et al. 2006; Visnjic and Banfic 2007). In fact, several lines of evidence have shown that nuclei contain a PIP_2-based signaling system that is distinct from that in the cytoplasm (Smith and Wells 1983; Cocco et al. 1987; Divecha et al. 1991; Martelli et al. 1991). The nuclear inositol lipids were found on structures called speckles inside the nucleus and virtually not in the NE. However, it is possible that invaginations of the NE could be enriched in PIP_2 such that it appears to be intranuclear (reviewed in Irvine 2006).

Several reports have studied the intranuclear regulation of phosphoinositides and their involvement in the progression of the cell cycle. For example, generation of DAG from PIP_2 during G2–M phase recruits PKCβII to the nucleus, where it phosphorylates laminins and participates in the regulation of NE breakdown (Fields et al. 1990; Hocevar and Fields 1991; Goss et al. 1994; Thompson and Fields 1996; Avazeri et al. 2000). Furthermore, IP_3 released in the nucleus may be phosphorylated to higher inositol phosphates (York et al. 1999; Odom et al. 2000; Shen et al. 2003; Steger et al. 2003), which in turn might have functions in messenger RNA export, transcriptional regulation, and/or chromatin structure. Moreover, the mitogenic action of insulin-like growth factor 1 is mediated by the phosphorylation of nuclear PLCβ1 by ERKs/mitogen-activated protein kinases (MAPKs) (Xu et al. 2001).

Further studies have, in fact, reported that receptors for several growth factors travel to the nucleus on stimulation (for a review, see Krolewski 2005), and importantly, they suggest that these receptors have this behavior in order to regulate cell proliferation responses. For instance, there is evidence that c-Met, the HGF receptor, translocates to the nucleus on HGF stimulation in liver cells in order to mediate nuclear Ca^{2+} signals dependent on hydrolysis of nuclear PIP_2 and on the

Table 5.1 Main components of the nuclear envelope (*NE*) membrane and their respective functions

Macromolecule	Function	Described in	Type	References
Adracalin	Unknown function	*Drosophila melanogaster*, *Saccharomyces cerevisiae*, and *Caenorhabditis elegans*	Protein	Cronshaw and Matunis (2003)
ANC-1	Important role in positioning the nucleus within the cytoplasm	*C. elegans*	Protein	Starr and Han (2002), Prunuske and Ullman (2006)
Ca^{2+} channels	Releases calcium for the fusion of nuclear vesicles	Chromaffin cells	Protein	Huh and Yoo (2003), Yoo et al. (2005)
Calreticulin	Lectin-like molecular chaperone for the folding of newly synthesized proteins and glycoproteins	CHO-K1 cells	Protein	Prunuske and Ullman (2006), Sugahara et al. (2009)
Diacylglycerol	Participation in the nuclear calcium signaling pathway	Swiss 3T3 cells	Lipid	Divecha et al. (1991), Martelli et al. (1992)
Emerin	A member of a family of type II integral membrane proteins which associates with the nuclear lamina, including lamina-associated proteins	COS-7 cells	Protein	Manilal et al. (1998), Prunuske and Ullman (2006)
GM1	Composes the Na^{+}/Ca^{2+} exchanger/ GM1 complex. Mediates transfer of nucleoplasmic Ca^{2+} to the NE lumen and hence to the endoplasmic reticulum and the nucleoplasmic reticulum, with which it is continuous.	Jurkat cells NG-CR72 cells	Ganglioside	Xie et al. (2002), Ledeen and Wu (2011)
Inositol 1,4,5-trisphosphate receptor	Functions as a Ca^{2+} release channel in response to intranuclear inositol 1,4,5-trisphosphate	SKHep1 cells	Protein	Echevarria et al. (2003)
Lamin B receptor	Provides attachment sites for heterochromatin and the nuclear lamina, the latter being a meshwork of intermediate filaments that associates with interphase chromatin and lines the inner nuclear membrane	COS-7 cells	Protein	Foisner and Gerace (1993), Prunuske and Ullman (2006)

(continued)

Table 5.1 (continued)

Macromolecule	Function	Described in	Type	References
Lamina-associated protein 2	Binds to lamins and interacts with chromatin through barrier-to-autointegration factor	CHO cells NRK cells Rat fibroblasts	Protein	Foisner and Gerace (1993), Prunuske and Ullman (2006)
LUMA	Likely interacts with the nuclear lamina	COS-7 cells	Protein	Dreger et al. (2001)
MAN1	Binds to lamins and interacts with chromatin through barrier-to-autointegration factor	HeLa cells	Protein	Paulin-Levasseur et al. (1996), Hetzer (2010)
Mannose N-linked oligosaccharides	Structural and recognition units	HeLa cells	Oligosaccharide	Fricker et al. (1997), Prunuske and Ullman (2006)
Metabotropic glutamate receptor 5	Mobilizes nuclear Ca^{2+} independent of cytosolic Ca^{2+} regulation	HEK cells Primary striatal neuronal cultures	Protein	O'Malley et al. (2003), Jong et al. (2005)
mrtl (*myc*-related translation/localization regulatory factor)	Functions primarily in the nucleus as a transcription factor for each of the three RNA polymerases	Normal primary human mammary epithelial cells	Protein	Choi et al. (2008)
Na^{+}/Ca^{2+} exchanger	Mediates transfer of nucleoplasmic Ca^{2+} to the NE lumen and hence to the endoplasmic reticulum, with which it is continuous	C6 cells	Protein	Xie et al. (2004), Ledeen and Wu (2011)
Nuclear pore complexes	Allows the passive diffusion of metabolites and proteins smaller than 40 kDa, and facilitates or activates transport of larger macromolecules	Eukaryotes	Protein	Cole and Hammell (1998), Wente and Rout (2010)
Nurim	Unknown function	Human osteosarcoma cell line U2OS	Protein	Hetzer (2010)
Protein kinase C	Participation in the nuclear calcium signaling pathway	Swiss 3T3 cells	Protein	Divecha et al. (1991), Martelli et al. (1992)
Protein disulfide isomerase	Enzymatic chaperone for reconstructing misfolded proteins in the endoplasmic reticulum lumen	HeLa cells	Protein	Prunuske and Ullman (2006), Wang et al. (2012b)

(continued)

Table 5.1 (continued)

Macromolecule	Function	Described in	Type	References
Ryanodine receptor	Regulates Ca^{2+} signals	C2C12 cells, a skeletal muscle derived cell line	Protein	Marius et al. (2006)
Sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase	Involved in regulating nucleoplasmic Ca^{2+} concentrations of vascular smooth muscle cells	Vascular smooth muscle cells	Protein	Abrenica et al. (2003), Ljubojevic et al. (2011)
Sec61p	Possible retrotranslocation channel for misfolded proteins	Mammalian and yeast cells	Protein	Schafer and Wolf (2009), Harsman et al. (2011), Malhas et al. (2011)
SREBP1	Controls transcription of the low density lipoprotein receptor gene	Fibroblasts	Protein	Yokoyama et al. (1993), Capanni et al. (2005)
SUN1 and SUN2	Interact with nesprins and establish connections with actin and intermediate filaments in the cytoplasm	*C. elegans* HeLa cells	Protein	Wilhelmsen et al. (2006), Hetzer (2010)
Cyclic ADP ribose	An enzyme involved in the biosynthesis of two Ca^{2+}-regulating second messengers, cyclic ADP ribose and nicotinic acid adenine dinucleotide phosphate	Hepatocytes *Aplysia* neurons	Protein	Khoo et al. (2000), Bezin et al. (2008)
S-100 Ca^{2+}-binding proteins	Characterized by a common structural motif, the EF-hand, have attracted major interest owing to their cell-specific and tissue-specific expression patterns and involvement in various pathological processes	Vascular smooth muscle cells and diverse other cell types	Protein	Mandinova et al. (1998), Hermann et al. (2012)

formation of nuclear IP_3 (Gomes et al. 2008). A similar study demonstrated that the insulin receptor translocates to the nucleus of hepatocytes on insulin stimulation to initiate IP_3-dependent Ca^{2+} signals there (Rodrigues et al. 2008). Since nucleoplasmic rather than cytosolic Ca^{2+} is essential for cellular proliferation, and is necessary in particular for progression through early prophase in liver cells (Rodrigues et al. 2007), it is thought that these growth factors may stimulate cell proliferation by selectively inducing Ca^{2+} signals in the nucleus.

In addition to growth factor receptors, several GPCRs have been found in the nucleus in various cell types (reviewed in Gobeil et al. 2006). For instance, the metabotropic glutamate receptor (mGlu5R) has been found in the nucleus of neurons (O'Malley et al. 2003) and HEK293 cells, where it couples to $G_{q/11}$ to activate PLC, generate IP_3, and mobilize nuclear Ca^{2+} (Kumar et al. 2008). There is considerable debate as to how a cell membrane receptor can reach the NE and the nucleus, or why a cell membrane receptor which is activated by extracellular ligands would be mobilized to the NE. A number of reviews have discussed these issues in reasonable detail and have suggested possible mechanisms; therefore, we do not address them here (Wells and Marti 2002; Carpenter 2003; Clevenger 2003; Johnson et al. 2004; Krolewski 2005). However, one could also question the mechanism by which extracellular ligands could act on nuclear receptors activating their intrinsic machinery to mobilize local nuclear Ca^{2+}. Jong et al. (2005) suggested that extracellular and polar ligands such as glutamate and quisqualate reach nuclear receptors via both sodium-dependent transporters and cystine glutamate exchangers. These two transporters were also identified in the NE (Wu et al. 2009). In cardiomyocytes, angiotensin receptor types 1 and 2 are localized both in the cell membrane and in the NE. Angiotensin II activation of receptors in the NE leads to an increase in NF-κB messenger RNA expression, and stimulation of angiotensin receptor type 1 evoked sustained Ca^{2+} concentration elevations in isolated nuclei through activation of the IP_3R. Angiotensin II can be produced in situ in intact cardiomyocytes and activates this intranuclear pathway (Gomes et al. 2010; Tadevosyan et al. 2010). The identification of the nucleoplasmic reticulum clarified this issue, since it was demonstrated for the first time that there is regulation of Ca^{2+} signaling in the nucleus independently from the cytosol in the liver cell line HepG2; ATP provoked Ca^{2+} concentration elevation earlier and at higher levels in the nucleus than in the cytosol. Moreover, nuclear IP_3R channels were more sensitive to IP_3 than the cytoplasmic ones. (Echevarria et al. 2003; Leite et al. 2003). Furthermore, Ca^{2+} release in the nucleus was shown to cause nuclear PKC to translocate to the NE, demonstrating a specific effect of localized nuclear Ca^{2+} signals. In isolated nuclei from liver cells, IP_3 or cADPR leads to an elevation of intranuclear Ca^{2+} concentration (Gerasimenko et al. 1995). Moreover, other second messengers such as NAADP, cADPR, and IP_3 were demonstrated to release Ca^{2+} from the NE of isolated pancreatic acinar nuclei, evoking a transient rise in the nucleoplasmic Ca^{2+} concentration (Gerasimenko et al. 2003). Additional studies demonstrated that stimulation of ventricular myocytes with endothelin 1 induced local release of Ca^{2+} from the NE via IP_3R type 2. This local Ca^{2+} release triggers histone deacetylase 5 phosphorylation and nuclear export,

which do not occur during the global Ca^{2+} transients at each heartbeat (Wu et al. 2006b). Subsequent studies showed that IP_3-induced Ca^{2+} release from the NE in fact generates Ca^{2+} transients in the nucleoplasm of cardiomyocyte isolated nuclei (Zima et al. 2007), and of electrically stimulated atrial myocytes (Kockskamper et al. 2008). The elevation of nuclear Ca^{2+} concentration induced by endothelin 1 in cardiomyocytes is involved in the pathway leading to hypertrophic cardiac growth (Higazi et al. 2009; Arantes et al. 2012).

5.6 Ca^{2+} Signaling in the Nucleus

5.6.1 IP_3Rs as a Target of Cell Death

IP_3-induced release of Ca^{2+} from the ER generates high-Ca^{2+} microdomains (range from 50 to 100 mM) at the tight ER–mitochondrial junctions, which activates the low-affinity mitochondrial Ca^{2+} uniporter, leading to mitochondrial Ca^{2+} uptake (Rizzuto et al. 1998; Csordas et al. 2010; Giacomello et al. 2010). This process of ER–mitochondria communication through IP_3R-mediated Ca^{2+} release may cause mitochondrial Ca^{2+} overload and it is linked to multiple models of apoptosis (Mikoshiba 2007). In fact, cells that do not express IP_3R are more resistant to apoptosis (Khan et al. 1996; Jayaraman and Marks 1997; Sugawara et al. 1997). Moreover, buffering of mitochondrial calcium leads to decreased apoptosis in hepatocytes (Guerra et al. 2011).

Currently, there is evidence that indicates the importance of IP_3R types 1 and 3 in the regulation of Ca^{2+}-dependent cell death (Boehning et al. 2005; Li et al. 2007; Eckenrode et al. 2010). Additionally, the interaction between the antiapoptotic proteins Bcl-2, Bcl-x_L, and Mcl-1 and the ER-localized IP_3Rs enables them to be fully efficacious as antiapoptotic mediators (Foyouzi-Youssefi et al. 2000). Stable expression of these three antiapoptotic proteins lowered the ER Ca^{2+} content and enhanced the rate of IP_3-mediated Ca^{2+} release in response to submaximal IP_3 stimulation in permeabilized wild-type DT40 cells but not in cells lacking all three types of IP_3R. In addition, expression of Bcl-2, Bcl-x_L, or Mcl-1 enhanced spontaneous IP_3R-dependent Ca^{2+} oscillations and spiking in intact cells in the absence of agonist stimulation (Eckenrode et al. 2010; Liu et al. 2012). Importantly, their function is regulated by posttranscriptional modifications. In particular, IP_3R phosphorylation appears to be a key common feature for modulation of channel function and, as a consequence, apoptotic signaling (Khan et al. 2006; Szado et al. 2008; Vanderheyden et al. 2009). Accordingly, Bcl-2 family members regulate IP_3R type 1 phosphorylation, controlling the rate of Ca^{2+} leak from ER stores and, thus, the apoptotic response (Oakes et al. 2005).

Furthermore, it was demonstrated that IP_3R type 3, localized in mitochondria-associated membranes, a physical domain of the ER platform for the interplay between the ER and mitochondria, has a selective role in the induction of apoptosis by preferentially transmitting apoptotic Ca^{2+} signals to mitochondria

(Blackshaw et al. 2000; Mendes et al. 2005). In a similar way, IP_3R type 3 silencing or knockout in mice blocked apoptosis and significantly decreased agonist-induced mitochondrial Ca^{2+} uptake (Blackshaw et al. 2000; Hayashi and Su 2007). In some cancer cells in which PKB is constitutively active (e.g., prostatic carcinoma cells), IP_3Rs are hyperphosphorylated (Khan et al. 2006), and consequently, ER Ca^{2+} release is inhibited significantly, reducing cellular sensitivity to Ca^{2+}-mediated proapoptotic stimulation (Marchi et al. 2008; Szado et al. 2008).

Accordingly, the tumor suppressor promyelocytic leukemia (PML) was found to physically interact with IP_3R type 3, modulating its phosphorylation state in a way similar to that in which Bcl-2 does with IP_3R type 1. PML is critical in multiple apoptotic pathways. The absence of PML inhibits cell death induced by various apoptotic stimuli. PML is critical for both transcription-dependent responses (e.g., p53-mediated responses) and transcription-independent early apoptotic responses (Salomoni and Pandolfi 2002; Bernardi et al. 2008).

Similarly, in *Pml* knockout mouse embryonic fibroblasts cells, IP_3R type 3 was demonstrated to be hyperphosphorylated (Giorgi et al. 2010), which was mediated by a specific multiprotein complex which included PML, IP_3R type 3, PP2A, and PKB, localized at ER–mitochondria-associated membrane contact sites. Moreover, PML seems to play a connective role between PP2A and IP_3R type 3, hence favoring IP_3R type 3 dephosphorylation. Thus, when PML is absent, PP2A is inactive, which leads to IP_3R type 3 hyperphosphorylation and reduced Ca^{2+} flux from the ER to mitochondria, rendering cells resistant to apoptotic Ca^{2+}-dependent stimuli (Giorgi et al. 2010; Bononi et al. 2011).

5.6.2 Effect of Nucleoplasmic Calcium on Cell Proliferation

Ca^{2+} signaling regulates innumerous functions in every cell, including proliferation, DNA condensation, anaphase onset and cytokinesis (Wolniak et al. 1983; Keith et al. 1985; Poenie et al. 1985; Twigg et al. 1988), and development and differentiation (Santella et al. 1998; Parry et al. 2005; Gomes et al. 2008; Resende et al. 2010a, b). It has also been related to plasticity of neurons and muscles (Carrasco et al. 2003; Greer and Greenberg 2008). Indeed, Ca^{2+} signals play an essential role in the progression of the cell cycle, specifically in the process of NE breakdown during mitosis (Moscat et al. 2003; Mellstrom et al. 2008).

Regulation of such physiological processes is achieved through the modulation of diverse gene networks, which in turn are controlled by transcription factors that translocate from the cytosol to the nucleus on changes of intracellular Ca^{2+} concentrations. NF-κB and NFAT are examples of such transcription factors (Schmidt et al. 2011).

In baseline conditions, NF-κB is anchored (inactive) in the cytoplasm by IκB (inhibitor of κB). IκB interacts with multiple ankyrin repeat motifs in NF-κB, which masks its nuclear localization signals (NLS), thus inhibiting nuclear translocation. In contrast, IκB kinase (IKK2) phosphorylates and inhibits IκB, which allows IκB to dissociate from NF-κB, exposing its NLS. IKK2 is activated either by Ca^{2+}/

CaM-dependent protein kinase (CaMK; type I, II, or III) (Theatre et al. 2009) or by PKC (Moscat et al. 2003; Mellstrom et al. 2008). Thus, Ca^{2+} transients trigger a phosphorylation cascade which culminate in activation of NF-κB and its translocation to the nucleus, where it activates the transcription of certain gene networks generally related to inflammatory or immune responses, cell survival responses, or cell proliferation. These gene networks modulate several Ca^{2+}-inducible signaling pathways, including CaM, CaMKII, and calcineurin, which are key proteins involved in cell cycle progression. CaM expression is increased during the G1–S transition (Chafouleas et al. 1982, 1984; Kahl and Means 2003), and manipulation of CaM levels disrupts the G1–M progression and interferes with DNA replication, thus affecting cellular growth and proliferation (Rasmussen and Means 1989; Shapiro et al. 1998; Yu et al. 2005). Both CaM and extracellular Ca^{2+} levels regulate the cell cycle machinery through the phosphorylation state of the tumor suppressor Rb (Takuwa et al. 1993; Knudsen et al. 1998). When dephosphorylated, Rb interacts with E2F transcription factors and prevents transcription of genes required for progression through the cell cycle. In contrast, when phosphorylated by cyclin-dependent kinases Cdk4 and Cdk2, Rb no longer interacts with E2F, and the cell cycle proceeds through the G1–S checkpoint (Hallstrom and Nevins 2009). Moreover, in agreement with the effects of CaM on the cell cycle, inhibition of CaMKII results in disruption of G2–M and G1–S progression (Kahl and Means 2003; Choi and Husain 2006). Furthermore, the cell cycle is arrested at the G2 phase with the enhanced expression of a constitutively active CaMKII (Planas-Silva and Means 1992; Patel et al. 1999). Likewise, progression through mitosis is defective in yeast mutants defective in CaM (Davis 1992; Ohya and Botstein 1994).

An additional family of transcription factors that regulate cell cycle is the NFAT family, which is involved in the control of the immune response and in the development of the heart, skeletal muscle, and the nervous system. Similar to NF-κB, NFAT is cytoplasmic in the resting cell. In its inactive state, NFAT has its NLS covered by phosphorylation groups. During cell activation, calcineurin, a Ca^{2+}/CaM-dependent phosphatase, dephosphorylates NFAT, resulting in NLS exposure, which leads to translocation of NFAT to the nucleus, allowing binding to the *cis*-regulatory elements of its target genes (Fig. 5.3) (Graef et al. 1999). Balance of activated/nonactivated calcineurin is maintained through kinases in the cytosol and by export kinases in the nucleus. Small and continuous cytoplasmic Ca^{2+} transients are sufficient for NFAT activation owing to the high affinity of calcineurin for Ca^{2+} (Teruel et al. 2000). However, a high amplitude of Ca^{2+} transients is necessary for NF-κB activation (Dolmetsch et al. 1997). Ca^{2+} oscillations are a more efficient stimulus than sustained Ca^{2+} concentration changes for NFAT activation in Jurkat T cells (Dolmetsch et al. 1998), undifferentiated stem cells (Kawano et al. 2006), or immature monocyte-derived dendritic cells (Vukcevic et al. 2010). In contrast, amplification of Ca^{2+} signals by SOCE (a process in which ER Ca^{2+} depletion induces influx of Ca^{2+} from the extracellular space) is essential for NFAT activation in diverse cell types (Oh-hora 2009; Courjaret and Machaca 2012).

Cytostatic factor (CSF) is an additional regulator of the cell cycle (Schmidt et al. 2006). Its cytoplasmic biochemical activity arrests the cell cycle at metaphase

of meiosis II during fertilization of vertebrate eggs. CSF prevents the activation of the anaphase-promoting complex/cyclosome (APC/C), keeping Cdk1 (also known as Cdc2) activity at a constant, high level. CSF is thought to suppress cyclin B degradation through the inhibition of APC/C–Cdk1, whereas cyclin B synthesis continues in unfertilized eggs (Yamamoto et al. 2005), inhibiting the transition to anaphase (Tunquist and Maller 2003). Fertilization elicits a Ca^{2+} transient that activates CaMKII (Lorca et al. 1993), which in turn phosphorylates the APC/C inhibitor Emi2 (Rauh et al. 2005; Hansen et al. 2006). Phosphorylated Emi2 becomes a substrate for phosphorylation by polo-like kinase. Dual phosphorylation of Emi2 targets it for degradation by the proteasome, thus releasing APC/C inhibition and releasing the metaphase II arrest (Schmidt et al. 2005).

However, depletion of the Emi2 protein from cycling egg extracts does not prevent mitotic cell cycle progression (Liu et al. 2006). Calcineurin has also been implicated in the metaphase II arrest release and in the APC/C activation during the meiotic cell cycle (Mochida and Hunt 2007; Nishiyama et al. 2007) through Emi2 dephosphorylation. Calcineurin inhibition has also been reported to arrest the cell cycle in the G1 phase (Schneider et al. 2002). Furthermore, antigen stimulation activates Ca^{2+} transients through SOCE, leading to calcineurin activation and resumption of the cell cycle (reviewed in Hogan et al. 2010). Accordingly, in different cell types, including RB1 mast cells, vascular smooth muscle, neuronal cells, and possibly other cells, activation of the c-*fos* gene is only achieved by Ca^{2+} oscillations with SOCE activation (Pulver et al. 2004; Di Capite et al. 2009). Local Ca^{2+} influx without global oscillations is enough to trigger gene activation in these cases (Di Capite et al. 2009), suggesting that the essential signal is an increase of the subplasmalemmal Ca^{2+} concentration (Kar et al. 2011). This situation is reminiscent of selective activation of certain genes by Ca^{2+} entry through L-type Ca^{2+} channels in neurons, vascular smooth muscle, and cardiomyocytes (Perez-Reyes 2003).

Gene expression may be directly controlled by L-type voltage-gated calcium channels through a signaling mechanism in which a C-terminal fragment of (calcium channel activated transcriptional regulator (CCAT) translocates to the nucleus in a calcium-dependent manner and regulates transcription (Chung and Jan 2006; Gomez-Ospina et al. 2006). Transcription is also regulated indirectly by CCAT through interaction with specific nuclear proteins in neurons (Gomez-Ospina et al. 2006) and in atrial cardiomyocytes (Schroder et al. 2009). Cleavage of CCAT from L-type voltage-gated calcium channels is dependent on the number of channels per cell and is performed by a constitutive proteolytic activity; this process is not regulated by Ca^{2+} (Gomez-Ospina et al. 2006). On the other hand, nucleoplasmic Ca^{2+} transients induce CCAT to dissociate from DNA and lead to its translocation to the cytoplasm (Chung and Jan 2006; Gomez-Ospina et al. 2006). Therefore, CCAT activates target genes, bypassing adaptor proteins and kinase cascades.

Cytosolic Ca^{2+} transients also induce shuttling of PLCδ1 to the nucleus, where it can induce the production of IP_3 and DAG (as discussed earlier), thus affecting several nuclear functions (Okada et al. 2002; Stallings et al. 2005). Similarly, cytosolic Ca^{2+} transients also lead to translocation of ADP ribose cyclase (CD38—an

enzyme involved in the biosynthesis of the Ca^{2+}-regulating second messengers cADPR and NAADP) to the nucleus (Bezin et al. 2008).

The cAMP response element binding (CREB) protein is another target of nucleoplasmic Ca^{2+} for cell proliferation (Hardingham et al. 1997). This transcription factor binds to DNA cAMP response element (CRE) sequences to regulate downstream gene transcription. CREB is known to regulate neuronal plasticity and long-term memory formation in the brain (Dolmetsch et al. 2001; Hardingham et al. 2001; Greer and Greenberg 2008). Some of the genes regulated by CREB are c-*fos*, the neurotrophin brain-derived neurotrophic factor, and many peptidic neurotransmitters. Coactivators of the CREB pathway include CREB binding protein (CBP) and transducers of regulated CREB proteins (TORCs). CREB is activated by phosphorylation via PKA, PKC, the mitogen-activated protein kinases (MAPK; ERK and p38), or CaMK (Fig. 5.4) (Siu et al. 2008).

CREB is activated through local cytosolic Ca^{2+} transients close to the L-type voltage-gated calcium channels, which activate CaMK and translocate it to the nucleus (Wheeler et al. 2008). CREB activity is also enhanced by nucleoplasmic Ca^{2+} transients. CREB binds to CRE together with CBP and TORCs, switching the downstream gene on or off. Nucleoplasmic Ca^{2+} transients also stimulate the binding of CBP and TORC (Hardingham et al. 1997, 2001) (for a review, see Mellstrom et al. 2008). CREB is dephosphorylated by protein phosphatase 1, which is activated by calcineurin, and terminates CRE-dependent transcription. Ca^{2+} transients and transcription activation are closely related in sympathetic neurons. Ca^{2+}/CaMKII acting near the L-type voltage calcium channels couples local Ca^{2+} concentration rise to CREB signal transduction. Like CREB phosphorylation, local CaMKII activity requires L-type channel gating, yet is only mildly dependent on the magnitude of Ca^{2+} flux. This is a complex coupling mechanism in which depolarization leads to CREB phosphorylation, and this process is encoded in the frequency of Ca^{2+} channel openings rather than the integrated Ca^{2+} flux (Wheeler et al. 2008). Different receptor channels may have a similar mechanism. Another excitation–transcription coupling to gene expression is demonstrated by glutamate channels and kinases other than CaMKII (e.g., MAPK) (Dolmetsch et al. 2001; Redmond et al. 2002; Wang et al. 2007). On the other hand, extrasynaptic glutamate receptors may shut down CREB activity (Vanhoutte and Bading 2003).

During development, neural stem cells display spontaneous calcium oscillations generated at the G1–S transition point through activation of the IP_3R and the RyR (Resende et al. 2010a). A connection between the frequency of spontaneous calcium oscillations, the activity of cell cycle mediators, and the duration of the G1 phase in neural stem cells has been demonstrated (Santos et al. 2012). Calcium imaging techniques confirmed that an increased calcium oscillatory frequency correlates with downregulation of the cell cycle inhibitor p27kip1, an augmented proliferative potential, and, in accordance with previous evidence (Salomoni and Calegari 2010), a decrease in the duration of the G1 phase (Resende et al. 2010a).

Another nuclear protein involved in the regulation of cell proliferation is SHIP1 (Gloire et al. 2007). Nuclear SHIP1 functions may be the same as for

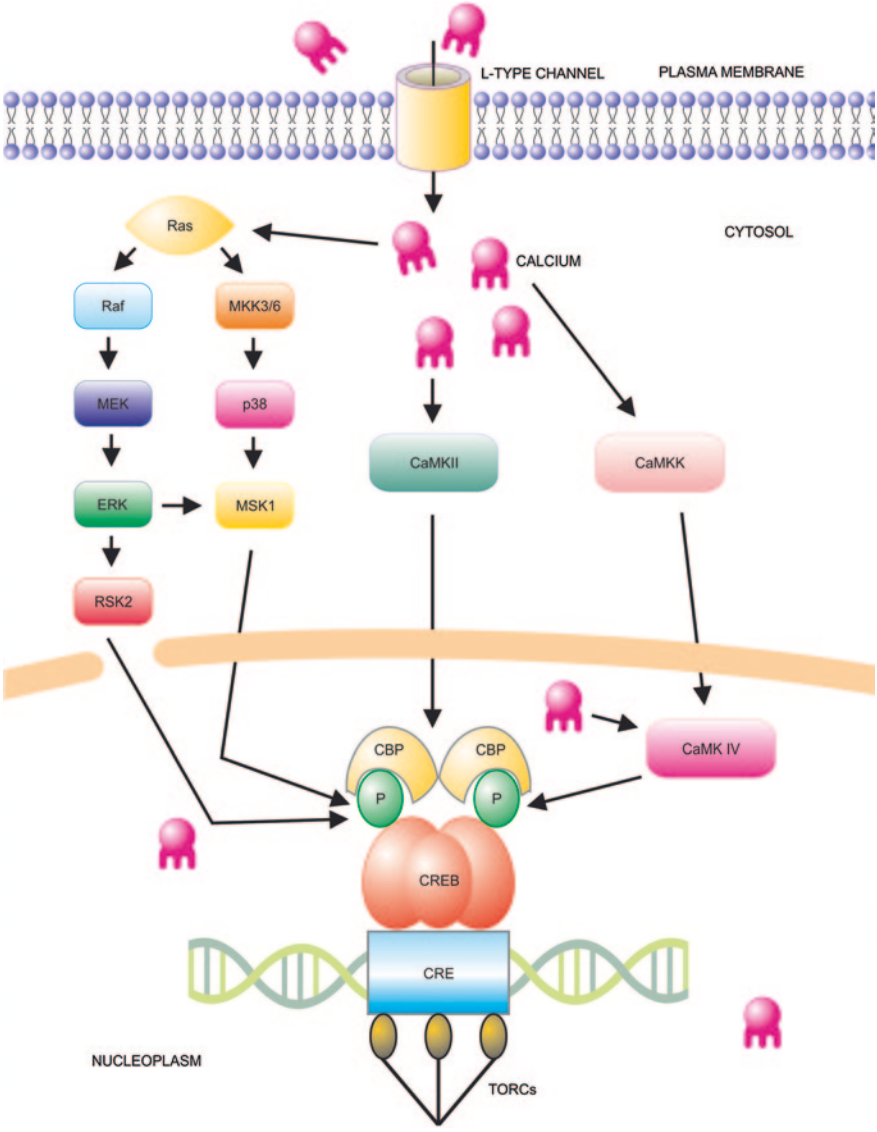

Fig. 5.4 Regulation of the transcription factor cyclic adenosine 3′,5′-monophosphate response element binding (*CREB*) protein by Ca^{2+}. CREB can be activated through local cytosolic Ca^{2+} transients close to the L-type voltage-gated calcium channels or by nucleoplasmic Ca^{2+} transients. In the cytosol, calcium promotes activation of several proteins involved in the phosphorylation cascades, leading to activation of CREB, for example, MKK (Ras; ERK and p38) or calcium/CaM-dependent protein kinases (*CaMK*). Coactivators of the CREB pathway include CREB binding protein (*CBP*) and transducers of regulated CREB proteins (*TORCs*). In the nucleus, Ca^{2+} activates CaMKIV, which promotes phosphorylation of CREB. Nucleoplasmic Ca^{2+} transients also stimulate the binding of CBP and TORC. The activation of CREB is related to cell proliferation. *CRE* cyclic adenosine 3′,5′-monophosphate response element

cytoplasmic SHIP1, a negative regulator of signaling processes by converting nuclear phosphatidylinositol 3,4,5-trisphosphate (PIP_3) to PIP_2 and/or nuclear inositol 1,3,4,5-tetrakisphosphate to inositol 1,3,4-trisphosphate. Nuclear SHIP1 may reduce the nuclear levels of PIP_3 alone or conjugated with nuclear phosphatase and tensin homologue (which converts PIP_3 to PIP_2), thus modulating PIP_3-dependent signaling in hematopoietic cells. PKB is an attractive target protein which may be regulated by the reduced level of nuclear PIP_3 produced by SHIP1-mediated modulation. PKB is activated when it is targeted to the plasma membrane and subsequently phosphorylated, a process mediated by binding of PIP_3 to the PKB pleckstrin homology domain. Thereafter, the isoforms PKBα and PKBβ translocate to the nucleus (Andjelkovic et al. 1997; Meier et al. 1997). Thus, it is possible that reduction of the levels of nuclear PIP_3 mediated by nuclear SHIP1 could lead to reduced levels of activated nuclear PKB isoforms. It has been suggested that nuclear SHIP1 may have a putative role in transcription as well. This hypothesis is supported by the fact that SHIP1-containing nuclear puncta partially colocalize with FLASH, a multifunctional nuclear protein that has been linked to apoptotic signaling and transcriptional control (Nalaskowski et al. 2012).

The myocyte enhancer factor 2 (MEF2) family includes several stress-response-related transcription factors that play an important role during embryonic development in several organs. MEF2 is normally repressed by a nucleoprotein complex that contains MEF2 binding domains. This complex competes with Ca^{2+}/CaM for binding to MEF2. Thus, nucleoplasmic Ca^{2+} transients activate MEF2 (Dolmetsch et al. 2001)

Nucleoplasmic Ca^{2+} can also interact with histones and high mobility group proteins to affect gene expression (Alonso and García-Sancho 2011). These proteins can be phosphorylated by CaMK and give rise to changes in chromatin structure that may expose or mask gene regulatory sites (Ding et al. 1994; Kardalinou et al. 1994). In addition, Ca^{2+}-dependent proteins such as CaM, S-100, and calreticulin are present in the nucleus, where they may be able to, directly or indirectly, interact with the chromatin (Mellstrom et al. 2008).

Together, these data show that Ca^{2+} signaling is essential during the progression of the cell cycle. In particular, nuclear Ca^{2+} acts through downstream Ca^{2+}-dependent signaling modules that regulate various aspects of cell cycle transition and cellular proliferation.

5.7 Conclusions

Ca^{2+} signaling results from the orchestrated and controlled action of the components of the Ca^{2+} signaling machinery that have been identified both in the cytosol and in the nucleus. In addition, the interaction of these with a myriad of intracellular and intranuclear players allows the performance of a wide variety of functions, in particular, cell proliferation. Understanding of the complexity of cytosolic and nuclear Ca^{2+} signals and their involvement in the progression through the cell cycle is indeed essential to, in turn, understand their physiological effects (e.g., normal cell

proliferation during development) as well as their pathological effects (e.g., abnormal cell proliferation in cancer). Further research on this topic is certainly needed and will potentially benefit many areas of the biological and medical fields.

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Chapter 6
Formation and Function of Lipid Droplets in Inflammation and Cancer

João P. B. Viola, André L. S. Cruz, Miriam B. F. Werneck and Patricia T. Bozza

Abstract Accumulation of lipid droplets (also known as lipid bodies or adiposomes) within leukocytes, epithelial cells, hepatocytes, and other non-adipocytic cells is a frequently observed phenotype in inflammatory conditions. Increasing evidence suggests that accumulation of these organelles is also a common feature of transformed cells and tissues. Lipid droplet biogenesis is a regulated cellular process, which culminates in the compartmentalization of lipids, which act as inducible organelles that play an important role in cell signaling, lipid metabolism, membrane trafficking, and control of the synthesis and secretion of inflammatory mediators. We review and discuss current evidence related to the biogenesis and function of lipid droplets in cell metabolism and signaling in inflammation and cancer. Moreover, the potential of lipid droplets as markers of disease and targets for novel anti-inflammatory and antineoplastic therapies is discussed.

Keywords Cancer • Inflammation • Lipid bodies • Lipid droplet • Lipid metabolism

6.1 Introduction

Lipid droplets are lipid-rich cytoplasmic organelles formed by a neutral lipid core surrounded by a monolayer of phospholipids composed of a diverse array of associated proteins, and may contain internal membranous structures (Martin and Parton 2006; Bozza et al. 2009b; Walther and Farese 2012). They are the main

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organelles involved in neutral lipid storage in eukaryotes, being constitutively present in fat-storing cells, including adipocytes and steroidogenic cells. Although lipid droplets are almost absent in most resting nonadipocytes, increased numbers are associated with inflammation and diseases such as cancer, in both experimental and clinical settings (Fig. 6.1). Studies are starting to shed light on the functions lipid droplets play in physiological and pathological conditions. The mechanisms that regulate the formation of lipid droplets and their functional significance for the cellular biology of inflammation and tumorigenesis are now under intense investigation. Although in the past the presence of cytoplasmatic lipid droplets was solely implicated in storage and lipid trafficking, it is now well established that these are highly regulated organelles involved in many aspects of cell activation and metabolism, and are important in inflammatory and neoplastic processes.

6.2 Increased Lipid Droplet Biogenesis Is Observed During Inflammation and Cancer

6.2.1 Lipid Droplets Are Endoplasmic-Reticulum-Derived Organelles

It has become increasingly evident that lipid droplet biogenesis involves specific and well-regulated mechanisms, and although the cellular and molecular mechanisms involved are still not completely understood, major advances have been made in recent years. The first and still largely accepted model of lipid droplet formation suggests the accumulation of neutral lipids within the double layer of the endoplasmic reticulum (ER) membrane, with subsequent budding off from the ER into the cytoplasm. A newly formed hydrophobic neutral lipid core surrounded by a monolayer of phospholipids directly derived from the cytoplasmic leaflet of the ER coated with proteins that lack transmembrane spanning domains detaches itself, creating an independent cytoplasmic organelle (Brown 2001; Murphy 2001; Robenek et al. 2004; Martin and Parton 2006; Brasaemle and Wolins 2012).

Although the prevailing hypothesis of lipid droplet biogenesis places lipid droplets as ER-derived organelles, different models of lipid droplet biogenesis have been proposed. Accumulating evidence obtained by different groups has suggested a greater complexity of lipid droplet structure and biogenesis than initially anticipated, and new hypothetical models of lipid droplet biogenesis have been proposed (Robenek et al. 2004; Ploegh 2007; Wan et al. 2007; Bozza et al. 2009b; Guo et al. 2009). Indeed, different studies reported the presence of membrane-associated and transmembrane spanning proteins (Dvorak et al. 1992, 1993, 1994; Bozza et al. 1997; Thore et al. 1998; Arend et al. 2004; Meadows et al. 2005; Robenek et al. 2005) as well as ribosomal structures, ribosomal associated proteins, and RNA-interacting proteins (Dvorak et al. 2003; Dvorak 2005) within lipid droplets in leukocytes and other cells. In addition, electron microscopy studies have reported images suggestive of

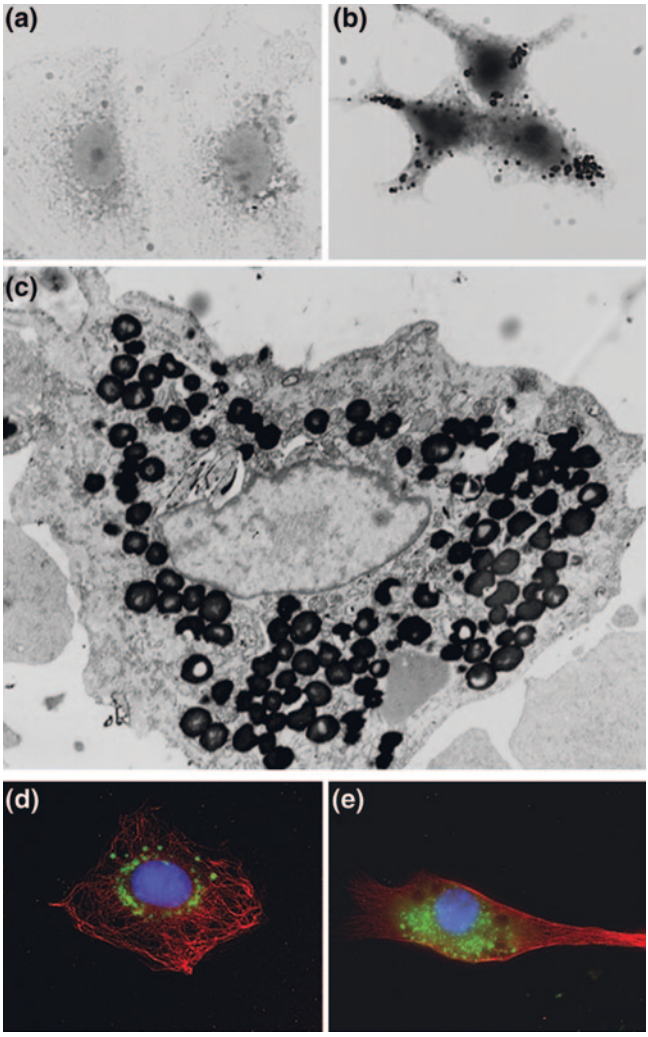

Fig. 6.1 Tumor and transformed cells accumulate lipid droplets in their cytoplasm. Immortalized, nontransformed rat IEC-6 colon epithelial cells (**a**) and human adenomacarcinoma cell line CACO-2 (**b**) stained with osmium tetroxide for visualization of lipid droplets. **c** Thin section of human colon cancer obtained at the time of surgery from a patient undergoing colon surgical resection, visualized by transmission electron microscopy. NIH 3T3 cells left untreated (**d**) or transfected to express H-*ras*V12 (**e**), a mutated version of H-*ras* that leads to transformation, were stained with BODIPY®, 4′,6-diamidino-2-phenylindole (DAPI), and phycoerythrin-conjugated antitubulin antibodies for visualization of lipid droplets, the nucleus, and the cytoskeleton , respectively

membranous structures as well as membrane "wrinkles" within lipid droplets, which allow the localization of proteins with predicted membrane insertion and transmembrane domains, including caveolin and cyclooxygenase (COX), to permeate lipid droplet cores (Dvorak et al. 1992, 1993, 1994; Bozza et al. 1997; Robenek et al.

2005; Walther and Farese 2009). These observations together with findings of enzymatic reactions localized in situ to lipid droplets, such as those observed for eicosanoid production discussed in Sect. 6.3, are suggestive of subcompartments within the lipid droplet, which are possible through the incorporation of ER-derived membranes within the forming lipid droplets (Wan et al. 2007; Bozza et al. 2009b).

6.2.2 *Specific Signaling Pathways and Cell-Dependent Mechanisms Are Involved in Lipid Droplet Formation*

Specific and well-regulated signaling pathways have been implicated in lipid droplet biogenesis in leukocytes and other cells involved in inflammatory and/or neoplastic reactions. Among the observations that indicate the existence of regulated production of lipid droplets in inflammatory cells are the findings that fully saturated fatty acids do not trigger lipid droplet formation, whereas *cis*-unsaturated fatty acids are potent inducers of lipid droplets (Weller et al. 1991b; Bozza et al. 1996a; Moreira et al. 2009), suggestive that lipid droplet formation involves more than simple incorporation of exogenous lipids. Accordingly, nonesterifiable *cis*-fatty acids such as the arachidonate analogue arachidonyl trifluoromethyl ketone are able to induce the formation of new lipid droplets (Bozza and Weller 2001). In addition, lipid droplet formation in macrophages is triggered by oxidized LDL, but not native LDL (Ross 1995; Li and Glass 2002; Schmitz and Grandl 2008). Moreover, stimulation with cytokines/chemokines and hormones induces receptor-mediated lipid droplet biogenesis not only in vivo but even in vitro in the absence of exogenous lipids (Bozza et al. 1998; Bandeira-Melo et al. 2001; Vieira-de-Abreu et al. 2005; Pacheco et al. 2007; Maya-Monteiro et al. 2008).

The signaling committed to lipid droplet biogenesis involves different pathways that are stimulus- and receptor-specific. Inflammatory lipid mediators, including platelet-activating factor (PAF) and PAF-like molecules (Bozza et al. 1996a, 1997, 1998; de Assis et al. 2003), but not lyso-PAF, and prostaglandin D_2 (Mesquita-Santos et al. 2006) acting via their specific G-protein-coupled receptor are potent inducers of lipid droplet formation. Other G-protein-coupled receptor agonists, including IL-8, C5a, and leukotriene B_4, did not induce leukocyte lipid droplet formation, demonstrating the requirement of specific intracellular signaling mechanisms in lipid droplet biogenesis (Bozza et al. 1996a). Lipopolysaccharide stimulation is also able to induce an increased number of lipid droplets in microglia through the activation of p38 and phosphatidylinositol 3-kinase (PI3K)/Akt pathways without activating JNK signaling (Khatchadourian et al. 2012). Leukocytes incubated with cytokines and chemokines even in the absence of exogenous lipids rapidly form new cytoplasmic lipid droplets by receptor-mediated processes (Bozza et al. 1998; Bartemes et al. 1999; Bandeira-Melo et al. 2001, 2002b; Pacheco et al. 2007). In human eosinophils, IL-5 alone or combined with granulocyte–macrophage colony stimulating factor, and stimulation with immobilized IgG induce significant increases in lipid droplet numbers (Bozza et al. 1998; Bartemes et al. 1999). Macrophage migration inhibitory factor and eotaxin

act in concert as key activators of the cells, eliciting induction of lipid droplets through cross talk between CD74 and CCR3 (Vieira-de-Abreu et al. 2011). CCR3-driven lipid droplet biogenesis was mediated by activation of mitogen-activated kinases, PI3K, and tyrosine kinases, whereas PAF effects involved protein kinase C (PKC) and phospholipase C downstream signaling (Bozza et al. 1996a; Bandeira-Melo et al. 2001).

The formation of lipid-droplet-enriched atherosclerotic activated macrophages, which differentiate to foam cells, involves complex and multistep mechanisms that depend on different signaling pathways regulating lipid influx, storage. and mobilization (Ross 1995; Li and Glass 2002; Schmitz and Grandl 2008). Different modifications of LDL, including enzymatic modification, acetylation, oxidation, and glycation, followed by recognition and activation of scavenger receptors, mostly CD36, play major roles in lipid accumulation in these cells (Goldstein et al. 1979; Brown and Goldstein 1983; Ross 1995; de Villiers and Smart 1999; Buechler et al. 2001; Kapinsky et al. 2001; Zhao et al. 2005; Rahaman et al. 2006; Hodgkinson et al. 2008; Schmitz and Grandl 2008). Modified LDL uptake by macrophages through scavenger receptors causes triglyceride and cholesterol loading, followed by cholesterol esterification mediated by acyl coenzyme A:acylcholesterol transferase and storage of cholesteryl esters in cytoplasmic lipid bodies (Ross 1995; Li and Glass 2002; Schmitz and Grandl 2008). In addition, different lipid-derived molecules generated in the process of LDL oxidation are involved in lipid body formation, including PAF-like molecules (Silva et al. 2002; de Assis et al. 2003), sterol ester (Chen et al. 2001), oxysterols (Leonarduzzi et al. 2005), 1-palmitoyl-2-(5′-oxovaleroyl)-*sn*-glycero-3-phosphocholine (Boullier et al. 2005), and azelaoyl phosphatidylcholine (de Assis et al. 2003). Monocyte chemoattractant protein 1 (MCP-1/CCL2), a key endogenous mediator involved in increased pathogenesis of macrophages in atherosclerosis due to recruitment and activation, is involved in the regulation of macrophage lipid droplet biogenesis in oxidized-LDL- and lipopolysaccharide-induced inflammation, as well as in experimental sepsis (Silva et al. 2002, 2009; Pacheco et al. 2007). MCP-1-driven lipid droplet accumulation is a highly regulated phenomenon dependent on MCP-1 receptor (CCR2) and downstream signaling through mitogen-activated protein kinases and PI3K (Pacheco et al. 2007). MCP-1-elicited lipid droplet assembly and protein compartmentalization was demonstrated to depend on a functional microtubule network. Accordingly, lipid droplets are enmeshed in a cytoskeleton network in several cell types (Franke et al. 1987; Dvorak 1991; Mermelstein et al. 2001; Wan et al. 2007). The lipid droplet–cytoskeleton interactions were shown to have roles in lipid droplet motility (Pol et al. 2004), rapid relocation on cell activation with chemotactic agents (Bandeira-Melo et al. 2001), and lipid droplet fusion and growth (Bostrom et al. 2005).

Adipokines, including leptin and resistin, were shown to modulate lipid droplet formation in macrophages and may participate in the mechanisms of foam cell formation (Xu et al. 2006; Maya-Monteiro et al. 2008; Maya-Monteiro and Bozza 2008). Interestingly, leptin-induced lipid droplet accumulation in macrophages in vivo or in vitro is accompanied by increased levels of adipose-differentiation-related protein (ADRP) (Maya-Monteiro et al. 2008). Increased ADRP expression by itself

has been shown to directly correlate with an enhanced capacity of neutral lipid storage, as ADRP promotes intracellular accumulation of triglycerides and cholesterol and reduces cholesterol efflux (Larigauderie et al. 2004). ADRP may also act as a nucleation center for the assembly of lipids to form nascent lipid droplets and to enhance droplet stability in lipolytic conditions (Wang et al. 2003; Gross et al. 2006).

Signaling control of protein synthesis and stability contributes to the dynamics of lipid droplet accumulation. The mammalian target of rapamycin (mTOR)-dependent signaling pathway was shown to be important in the translational control of lipid droplet biogenesis (Maya-Monteiro and Bozza 2008), since the numbers of leptin-induced ADRP-enriched lipid droplets were drastically reduced by treatment with the mTOR inhibitor rapamycin (Maya-Monteiro et al. 2008). Indeed, mTOR-dependent translational control of ADRP expression has been suggested in adipocytes stimulated with conjugated linoleic acid and in macrophages stimulated with leptin (Chung et al. 2005; Maya-Monteiro et al. 2008).

Transcription-dependent mechanisms are also involved in lipid droplet biogenesis. The best characterized mechanisms involve the activation of the transcription factors sterol regulatory element binding protein and peroxisome-proliferator-activated receptor (PPAR). PPARγ directly regulates the expression of several genes participating in fatty acid uptake, lipid storage, and the inflammatory response by binding to specific DNA response elements in target genes as heterodimers with the retinoid X receptors, including fatty acid synthase and ADRP (Gearing et al. 1993; Keller et al. 1993; Chawla et al. 2001b). Indeed, a role for PPARγ in lipid droplet biogenesis has been established in atherosclerotic and infection-triggered reactions (Nagy et al.1998; Ricote et al. 1998; Tontonoz et al. 1998; Chawla et al. 2001a; Almeida et al. 2009). Of note, treatment with the fatty acid synthase inhibitor C75 has been shown to significantly inhibit new lipid droplet formation in macrophages induced by apoptotic cells with or without infection (D'Avila et al. 2008), in cells infected with dengue virus (Samsa et al. 2009), and in cancerous cells (Accioly et al. 2008), confirming the role of new lipid synthesis in lipid droplet biogenesis.

Upregulated lipogenesis is a phenotype common to numerous human carcinomas and has been associated with poor prognosis in breast, prostate, and colon cancer (Kuhajda 2006; Swinnen et al. 2006). Two processes may be responsible for the increased numbers of lipid droplets observed in tumor cells: the association of transformed cells with adipose tissue surrounding the tumor site or necrotic parts within the tumor (Zoula et al. 2003; Moritani et al. 2011; Nieman et al. 2011); and intrinsic changes to lipid metabolism. Altered lipid metabolism in cancer cells involves modulation of numerous lipogenic enzymes (Kuhajda 2006; Swinnen et al. 2006; Wang et al. 2012), and culminates in the accumulation of newly formed lipids in cytoplasmic lipid droplets (Fig. 6.1). Indeed, enhanced numbers of lipid droplet have been described in several neoplastic processes, including adenocarcinoma of the colon (Accioly et al. 2008), invasive squamous cervical carcinoma (Than et al. 2003), human brain tumor (Opstad et al. 2008), hepatocarcinoma (Dvorak et al. 1993), and Burkitt lymphoma (Ambrosio et al. 2012) (Table 6.1), and may be directly associated with events of cellular transformation. Data from our laboratory have shown that oncogenic transformation of

Table 6.1 Tumors where altered lipid droplets or expression of lipid-droplet-associated proteins is observed

Tissue	Tumor type	PAT expression[a]	Lipid droplets	Phenotype changes	References
Brain	Human brain tumor	ND	+		Opstad et al. (2008)
Breast/ mammary gland	Apocrine carcinoma	Low TIP47	ND	ADRP-positive tumor cells preferentially facing adipose tissue	Straub et al. (2010)
		High ADRP	+		Moritani et al. (2011)
	Carcinoma of the breast	ND	+	Lipid droplet accumulation correlates with Her2 expression	Guan et al. (2011)
	Invasive ductal carcinoma	Low ADRP	−	ADRP positively correlated with increased proliferation	Straub et al. (2010)
		TIP47[b]			Mellick et al. (2002)
	Invasive lobular carcinoma	Low ADRP	−	ADRP positively correlated with increased proliferation	Straub et al. (2010)
	Tubular carcinoma	Low ADRP	−	ADRP positively correlated with increased proliferation	Straub et al. (2010)
Cervix	Cervical dysplasia	High TIP47	−	TIP47 positively correlated with disease severity	Szigeti et al. (2009)
					Than et al. (2003)
	Invasive carcinoma	High TIP47	−	TIP47 detected on serum when tumor became invasive and on recurrence	Szigeti et al. (2009)
					Than et al. (2003)
Colon	Adenocarcinoma	High ADRP, TIP47	+	TIP47 positively correlated with tumor size and higher tumor grade	Straub et al. (2010)
		High ADRP	+	ADRP positively correlated with increased proliferation and PGE_2 production	Accioly et al. (2008)
	Tubular adenoma	High ADRP, TIP47	−		Straub et al. (2010)
Kidney	Chromophobic renal cell carcinoma	ADRP, TIP47, PLIN	+		Straub et al. (2010)
	Clear cell renal carcinoma	ADRP, TIP47, PLIN	+	Increased TIP47 expression in metastasis	Straub et al. (2010)
		High ADRP[b]	−	ADRP correlated negatively with tumor grade	Rae et al. (2000)
		High ADRP[b]	−	ADRP correlated with good prognosis	Yao et al. (2005)
		High ADRP[b]	−	Lower ADRP expression in metastasis	Yao et al. (2007)
	Invasive renal squamous cell carcinoma	ND	+		Than et al. (2003)
	Kidney angiomyolipoma	ADRP, TIP47, PLIN	−		Straub et al. (2010)
	Papillary renal cell carcinoma	ADRP, TIP47, PLIN	+		Straub et al. (2010)

(continued)

Table 6.1 (continued)

Tissue	Tumor type	PAT expression[a]	Lipid droplets	Phenotype changes	References
Larynx	Laryngeal squamous cell carcinoma	ADRP, TIP47, PLIN	−		Straub et al. (2010)
Liver	Cholangiocarcinoma	High ADRP, low PLIN	NC		Straub et al. (2010)
	Dysplastic nodules	High ADRP, low PLIN	−		Straub et al. (2010)
	Focal nodular hyperplasia	High ADRP, PLIN Low TIP47	−		Straub et al. (2010)
	Hepatocellular adenoma	High ADRP, TIP47	−		Straub et al. (2010)
	Hepatocellular carcinoma, all grades	High ADRP, low PLIN High ADRP[b]	+	ADRP positively correlated with increased proliferation	Straub et al. (2010) Kurokawa et al. (2004)
	Hepatocellular carcinoma grade 1	High ADRP, low PLIN	−		Straub et al. (2010)
	Hepatocellular carcinoma grade 2	High ADRP, low PLIN	−		Straub et al. (2010)
	Hepatocellular carcinoma grade 3	High ADRP, low PLIN	−		Straub et al. (2010)
Lung	Large cell lung carcinoma	High ADRP,TIP47	+	TIP47 positively correlated with tumor size	Straub et al. (2010)
	Lung adenocarcinoma	High TIP47	+		Straub et al. (2010)
	Lung squamous cell carcinoma	High ADRP, TIP47	+	TIP47 positively correlated with tumor size	Straub et al. (2010)
	Sarcomatoid/pleomorphic lung carcinoma	Low ADRP, high TIP47	−		Straub et al. (2010)
Lymphoma	Burkitt lymphoma	ADRP[b]	+	Differential ADRP expression from diffuse large B cell lymphoma	Ambrosio et al. (2012)
Ovary	Ovarian adenocarcinoma	ND	+	Lipid accumulation correlated with increased proliferation and tumor size	Nieman et al. (2011)
Pancreas	Pancreas ductal adenocarcinoma	ADRP, TIP47, PLIN	−		Straub et al. (2010)
Prostate	Prostate gland adenocarcinoma	ADRP, TIP47, PLIN	NC		Straub et al. (2010)

(continued)

Table 6.1 (continued)

Tissue	Tumor type	PAT expression[a]	Lipid droplets	Phenotype changes	References
Skin	Actinic keratosis	Low ADRP,TIP47	−		Straub et al. (2010)
	Basal cell skin carcinoma	High ADRP, low TIP47	+		Straub et al. (2010)
		ADRP	−		Ostler et al. (2010)
	Sebaceous adenoma	High ADRP, PLIN	−		Straub et al. (2010)
		ADRP			Ostler et al. (2010)
	Sebaceous carcinoma	High ADRP, TIP47, PLIN	−		Straub et al. (2010)
		ADRP	+		Ostler et al. (2010)
		ADRP,TIP47, PLIN	+		Muthusamy et al. (2006)
	Skin carcinoma in situ	Low ADRP, TIP47	−		Straub et al. (2010)
	Skin squamous cell carcinoma	Low ADRP, TIP47	+	TIP47 positively correlated with tumor size	Straub et al. (2010)
		ADRP	−		Ostler et al. (2010)
Stomach	Stomach adenocarcinoma	ADRP, TIP47, PLIN	−		Straub et al. (2010)

ADRP adipose-differentiation-related protein, *NC* data presented by the authors do not allow a clear conclusion to be drawn, *ND* not determined, *PAT*, *PDE*$_2$ prostaglandin E$_2$, *PLIN* perilipin, *TIP47* tail-interacting protein of 47 kDa

[a]Increased or decreased expression when compared with nontumoral tissue/samples

[b]Alteration of messenger RNA levels

nontransformed rat intestinal epithelial cells (IEC-6 cells) by ectopic expression of a constitutively active H-*ras*V12 leads to highly increased lipid droplet biogenesis and enhanced prostaglandin E_2 (PGE_2) production when compared with parental IEC-6 cells (Accioly et al. 2008). Of note, stimulation of these nontransformed cells with mitogens and activating agents, including treatment with phorbol 12-myristate 13-acetate or with unsaturated fatty acids, also increased lipid droplet formation (Accioly et al. 2008; Moreira et al. 2009). In these models it is not clear if the increase in the numbers of lipid droplets is a direct consequence of signaling pathways activated by H-*ras*V12 or PKC/mitogen-activated protein kinase since cancer cells may also secret soluble factors that act in a paracrine fashion to induce lipid droplet formation. Conditioned medium from different human cancer cell lines, including human lung squamous carcinoma, adenocarcinoma, acute promyelotic leukemia, and human cervical epithelioid carcinoma cells, but not from nontransformed cells, was able to trigger lipid droplet formation in preadipocytes (Hirano et al. 2008). Conversely, it was recently suggested that adipocytes are able to induce lipid droplets in cancer cells because ovarian cancer cells in omental metastasis contained abundant lipids at the adipocyte–cancer cell interface (Nieman et al. 2011). Notably, co-culture of ovarian, breast, or colon cancer cells with adipocytes resulted in cytoplasmic lipid droplet accumulation in the transformed cells (Nieman et al. 2011). Moreover, fluorescent lipids were transferred from adipocytes to ovarian cancer cells during co-culture, supporting a model in which adipocytes provide lipids to support tumor growth (Nieman et al. 2011).

6.3 Functions of Lipid Droplets in Inflammation and Cancer

6.3.1 Lipid Droplets Are Sites for Eicosanoid Formation in Inflammation and Cancer

It is now becoming increasingly recognized that lipid droplets are specialized locales involved in compartmentalization and amplification of eicosanoid synthesis. Eicosanoids are a family of arachidonic acid derived signaling lipids that control important cellular processes, including cell activation, migration, proliferation. and apoptosis (Yaqoob 2003; Wymann and Schneiter 2008). Thus, eicosanoids have key roles in physiological and pathological conditions such as tissue homeostasis, inflammation, and cancer (Yaqoob 2003; Wymann and Schneiter 2008). Analyses of lipid droplets in different cell types and stimulatory conditions have demonstrated that lipid droplets are particularly active sites for the metabolism of arachidonyl lipids. Electron microscopic, autoradiographic, and subcellular fractionation observations demonstrated that arachidonate is incorporated and esterified predominantly in lipid droplets of leukocytes, epithelial cells, and neoplastic cells (Dvorak et al. 1983, 1993; Weller et al. 1989, 1991a; Plotkowski et al. 2008).

Free arachidonic acid is an extremely reactive molecule that functions in cell signaling as an intracellular second messenger, as a paracrine mediator of cell activation, and as a substrate for enzymatic conversion into eicosanoids (Six and Dennis 2000; Yaqoob 2003). Although, negligible amounts of free arachidonic acid were identified in lipid droplets, different enzymes involved in arachidonic acid metabolism were demonstrated to localize in lipid droplets, thus providing strong evidence for a major role of lipid droplets in arachidonic acid metabolism. Within the droplet, arachidonic acid is shielded and inert. To be accessible for metabolization and function as a signaling molecule, the arachidonic acid present in lipid droplets must be released by phospholipases; the free arachidonate then gains access to eicosanoid-forming enzymes. Cytosolic phospholipase A_2 ($cPLA_2$) specifically hydrolyzes arachidonic acid from the *sn*-2 position of glycerophospholipids and is the rate-limiting enzyme in the formation of eicosanoids and PAF (Ghosh et al. 2006). $cPLA_2$ and its activating protein kinases, ERK1 and ERK2, were demonstrated to co-localize at lipid droplets in cells responding to a wide range of stimuli, including arachidonic acid (Wooten et al. 2008; Moreira et al. 2009). Moreover, on subcellular fractionation, high $cPLA_2$ specific activity was enriched in the lipid-droplet-containing fraction (Yu et al. 1998).

Intracellular compartmentalization of eicosanoid synthesis has emerged as a key feature that regulates the amount and the type of eicosanoid produced. Accordingly, the co-localization of key eicosanoid-forming enzymes to these organelles in inflammatory and neoplasic conditions strongly supports a role for lipid droplets in the enhanced eicosanoid generation observed in these conditions. The major enzymes–5-lipoxygenase, 15-lipoxygenase, 5-lipoxygenase-activating protein (FLAP), and COX–involved in the enzymatic conversion of arachidonic acid to eicosanoids were shown to localize within lipid droplets by different cellular imaging techniques, as well as by Western blotting of subcellular fractions of lipid droplets after in vitro stimulation (Dvorak et al. 1992, 1993; Bozza et al. 1997, 1998; Accioly et al. 2008; Silva et al. 2009) or from in vivo inflammatory responses (Pacheco et al. 2002; Vieira-de-Abreu et al. 2005; D'Avila et al. 2006; Maya-Monteiro et al. 2008). Moreover, even the downstream eicosanoid-forming enzymes leukotriene C_4 synthase and PGE_2 synthase have been localized to lipid droplets (Bozza et al. 1997; Meadows et al. 2005; Accioly et al. 2008). Collectively, these observations suggest that in inflammatory and neoplasic conditions, lipid droplets actively compartmentalize the entire enzymatic machinery for eicosanoid synthesis, generating a niche for synthesis of inflammatory mediators.

Despite the strong correlation, efficient eicosanoid production is not determined only by the availability of arachidonic acid and eicosanoid-forming enzymes, as it requires sequential interactions between specific biosynthetic proteins acting in cascade, and may involve very unique spatial interactions. Therefore, just by detecting eicosanoid-forming enzymes within lipid droplets one cannot establish these organelles as accountable for the efficient and enhanced eicosanoid synthesis observed during inflammatory responses. In support of the roles of lipid droplets in eicosanoid formation, significant correlation between lipid droplet formation and enhanced generation of both lipoxygenase- and COX-derived eicosanoids

has been demonstrated in vitro (Bozza et al. 1996a, b, 1997, 1998; Bartemes et al. 1999; Weller et al. 1999; Pacheco et al. 2007) as well as in vivo (Pacheco et al. 2002; de Assis et al. 2003; Melo et al. 2003; Vieira-de-Abreu et al. 2005; D'Avila et al. 2006; Maya-Monteiro et al. 2008; Paiva et al. 2010), thus suggesting that increased lipid droplet numbers in cells would result in enhanced capacity of eicosanoid production. Generally, direct assessment of specific intracellular sites of eicosanoid synthesis has been elusive, as those lipid mediators are newly formed, not stored, and often rapidly released upon cell stimulation. By means of a recently developed technique, named Eicosa Cell, that uses a strategy to covalently cross-link, capture, and identify newly formed eicosanoids at their sites of synthesis (Bandeira Melo et al. 2011), it was established that lipid droplets are major intracellular locales for the activation-elicited formation of leukotriene C_4 in eosinophils (Bandeira-Melo et al. 2001, 2002a; Vieira-de-Abreu et al. 2005), leukotriene B_4 in neutrophils and macrophages (Pacheco et al. 2007; Monteiro et al. 2011), and PGE_2 in macrophages and epithelial cells (D'Avila et al. 2006; Accioly et al. 2008; Plotkowski et al. 2008; Moreira et al. 2009).

Importantly, eicosanoid formation within lipid droplets is not restricted to leukocytes or to inflammatory conditions. Cells that produce high quantities of eicosanoids under physiological conditions, including granulosa cells of periovulatory follicles, for which the PGE_2 produced is necessary for normal ovulation (Seachord et al. 2005), luteal-steroid-producing and interstitial cells involved in regression of the corpus luteum (Arend et al. 2004), and fetal membranes with advancing gestation and labor (Meadows et al. 2003, 2005), were demonstrated to exhibit high numbers of lipid droplets containing eicosanoid-synthesizing enzymes. Moreover, endothelial and epithelial cells involved in pathological conditions such as cancer, hypoxia, and during infections were shown to contain increased numbers of eicosanoid-synthesizing lipid droplets (Dvorak et al. 1992, 1993, 1994; Scarfo et al. 2001; Accioly et al. 2008; Plotkowski et al. 2008; Paiva et al. 2010). It is worth noting that lipid droplets are heterogenic and may vary in composition and function in response to different stimuli. In resting conditions, hepatic stellate cells exhibit high numbers of large lipid droplets that are involved in the storage of retinoids (vitamin A and its metabolites) and show undetectable level of leukotriene production (Borojevic et al. 1990; Guaragna et al. 1991; Friedman 2008; Blaner et al. 2009; Paiva et al. 2010). On infection with schistosomes, the retinoid storage ability of these organelles is lost (Borojevic et al. 1990; Guaragna et al. 1991; Friedman 2008; Blaner et al. 2009; Paiva et al. 2010), and the lipid droplets of schistosome-activated hepatic stellate cells become sites of 5-lipoxygenase compartmentalization and leukotriene formation (Paiva et al. 2010). In agreement with these observations and as discussed for the process of cellular transformation, lipid droplets contribute to eicosanoid production in response to pathogen infections in different cell types and pathological conditions (Pacheco et al. 2002, 2007; D'Avila et al. 2006, 2011; Sorgi et al. 2009; Mattos et al. 2010, 2011a, b). Altogether, these features highlight the plasticity of lipid droplets and suggest that the heterogeneity of this organelle's structure and function depend on and contribute to specific characteristics of the cell type, activation state, and inflammatory environment.

6.3.2 Lipid Droplets in Cell Metabolism and Proliferation

Recently published data have shown that an increase in lipid droplet numbers occurs in cells undergoing cell proliferation and that this is a common feature in many neoplastic processes (Bozza and Viola 2010). Although it has been suggested this may contribute to cell proliferation, no definitive studies are available that establish a causal link between the increase in lipid droplet numbers and development of cancer.

Of relevance to the roles of lipid droplets in cell metabolism and proliferation, a variety of signaling-associated proteins have been demonstrated to compartmentalize within lipid droplets, suggesting a key role for these organelles as a cytoplasmic hub favoring intracellular signaling. Indeed, proteins with well-established roles in the pathogenesis of inflammation and oncogenic cell transformation, tumorigenesis, and metastasis, including PI3K, ERK1, ERK2, p38, PKC, and caveolin, were shown to localize to lipid droplets in a variety of cell types (Yu et al. 1998, 2000; Fujimoto et al. 2001; Pol et al. 2001; Chen et al. 2002; Cohen et al. 2004). Mitogen-activated protein kinases ERK1, ERK2, and p38 are key enzymes in the activation of $cPLA_2$, the enzyme that specifically hydrolyzes arachidonic acid at the *sn*-2 position of glycerophospholipids. The substantial association of the $cPLA_2$ activators ERK1 and ERK2 with lipid droplets may contribute to efficient phosphorylation of $cPLA_2$ in these organelles in response to extracellular stimuli. PI3K regulatory and catalytic subunits were also localized to lipid droplets in a human histiocytic lymphoma cell line and in PAF-stimulated human neutrophils (Yu et al. 2000). Of note, co-immunoprecipitation studies demonstrated PI3K to be physically associated with phosphorylated Lyn kinase in lipid droplets of activated human neutrophils (Yu et al. 2000). Although functional studies still need to be performed to characterize the actual role of lipid-droplet-resident kinases, accumulating evidence indicates that kinase-mediated signaling is active within cytoplasmic lipid droplets in leukocytes and suggests they may act as a scaffold to favor quick intracellular signaling on cellular stimulation mediated by specific membrane receptors.

Potential functions of lipid droplets as sites of ribosomal translation and de novo protein synthesis have been proposed, with potential implications for the regulation of cancerous and inflammation-related processes. Electron microscopy analyses of lipid droplets demonstrated the presence of ribosomes or particles resembling ribosomal subunits in these organelles (Dvorak et al. 2003; Dvorak 2005; Wan et al. 2007). Moreover, ribosomes or ribosome-subunit-like particles were present within the lipid-rich cores and/or attached to the surface of lipid droplets in the human histiocytic lymphoma cell line U937 and in activated human neutrophils and eosinophils (Wan et al. 2007). The suggestion that lipid droplets are sites of ribosomal function is supported by the demonstration that ^{3}H-uridine accumulates and messenger RNA can be detected by in situ hybridization in these organelles (Dvorak et al. 2003; Dvorak 2005). Moreover, proteomic analyses of purified leukocyte-derived lipid-droplet-rich subcellular fractions identified several ribosomal subunit proteins as

well as translation initiation factors (Wan et al. 2007), an observation supported by proteomic analyses of lipid droplets from a hepatoma cell line expressing the capsid protein from hepatitis C virus (Sato et al. 2006). Further investigations are necessary to characterize the roles of lipid droplets in the regulation of local protein synthesis during inflammation and cell transformation.

The wide variety of signaling-associated proteins localized to lipid droplets suggests that these organelles may partake in cellular proliferation and differentiation. One of the possible mechanisms that remains to be addressed is in what manner lipid droplets contribute to cell cycle progression, and if lipid droplet biosynthesis or composition may play a role in uncontrolled proliferation that culminates in diseases such as cancer. The first mechanistic insights into how lipid droplets may participate in cell cycle regulation were provided by studies in budding yeast suggestive of a direct link between cell-cycle-regulatory kinases and lipid-droplet-derived triacylglycerol degradation (Kurat et al. 2009). It was observed that the yeast lipase Tgl4, an analogue of human adipose triglyceride lipase that co-localizes in lipid droplets, is a target for phosphorylation by the major yeast cell cycle regulator Cdc28 (human cyclin-dependent kinase 1 analogue) (Kurat et al. 2009). Absence of Cdc28-mediated phosphorylation leads to a loss of Tgl4 lipolyse activity and, consequently, a delay in bud formation and cell cycle progression. In addition, combined inhibition of both lipid hydrolysis and fatty acid production completely abolished progression through the G1 cell cycle phase (Kurat et al. 2009). Together, these data suggest the existence of a general mechanism in yeast that coordinates membrane synthesis with cell cycle progression. More recently, another work demonstrated that regulation of lipid droplets may be tightly coordinated with cell cycle progression in fission yeast. In this model, lipid droplet formation was observed by time-lapse confocal microscopy during the G2 cell cycle phase, divided between de novo biogenesis and a supposedly fission process of preexistent lipid droplets (Long et al. 2012). However, despite all promising data concerning lipid droplets and cell cycle progression, it is still unclear whether lipid droplets directly play a role in cell proliferation.

A positive correlation of increase in lipid droplet numbers and cell proliferation has been established in colon cancer cells (Accioly et al. 2008). Although it is not clear if it is cause or a consequence of the transformation process, a clear dependency of the maintenance of these lipid bodies for continued cellular proliferation was established. Accordingly, an association between lipid droplets and cell proliferation has also been observed in the regenerating liver of caveolin-1 knockout mice (Fernandez et al. 2006). Caveolin-1 belongs to a group of proteins involved in the formation of caveolae and lipid homeostasis, and was previously described as important for biogenesis of lipid droplets (Martin and Parton 2005). Hepatocytes from caveolin-1-deficient mice exhibit a reduced number of lipid droplets and cell cycle arrest at the G1 phase following partial hepatectomy, which impairs liver regeneration and reduces survival (Fernandez et al. 2006). Since glucose administration was able to reestablish cell cycle progression, it is possible that, in this system, caveolin-containing lipid droplets supply hepatocytes with energy to fuel their high metabolic rate, making possible proliferation (Brasaemle 2006; Fernandez et al. 2006).

6.4 Potential for Lipid Droplets as Disease Markers and Therapeutic Targets

As discussed already, lipid droplets may function as specialized intracellular sites of signaling within cells engaged in inflammatory process ranging from infections to atherosclerosis and cancer. Although no specific lipid droplet inhibitor has been described so far, different classes of drugs as well as gene knockdown of PAT proteins have been demonstrated to inhibit lipid droplet formation. The hypothesis of lipid droplet inhibition as a target for anti-inflammatory therapy has been tested in different model systems.

Inducible mechanisms that regulate eicosanoid production are attractive targets for pharmacological intervention. Aspirin and other selected nonsteroidal anti-inflammatory drugs (NSAIDs) inhibited lipid droplet formation in vivo and in vitro (Bozza et al. 1996b, 2002; Vieira-de-Abreu et al. 2005). However, the mechanisms involved in NSAID inhibition of lipid droplet biogenesis are not completely understood. Interestingly, it has been demonstrated that COX-independent mechanisms are involved in lipid droplet formation, since *cis*-unsaturated fatty acids induce lipid droplet biogenesis in macrophages that lack COX-1 and COX-2 expression; and NSAIDs, including aspirin, sodium salicylate, indomethacin, and NS-398, can inhibit lipid droplet formation in COX-1- or COX-2-deficient macrophages as well as in wild-type cells (Bozza et al. 1996b, 2002).

Clinical and experimental evidence strongly suggests that NSAIDs are anticarcinogenic, antiproliferative, and antineoplastic. In fact, the oncogenic potentials of the prostaglandins are related to their properties that promote cell proliferation and inhibit apoptosis in intestinal epithelial cells (Tsujii and DuBois 1995; Sheng et al. 1998, 2001). However, it is still not clear whether the antineoplastic effect of aspirin is attributable exclusively to its ability to inhibit prostaglandin production. A role for lipid droplets as a potential target to generate new drugs for cancer treatment has been suggested recently (Accioly et al. 2008). The human colon cancer cell line CACO-2 exhibits progressive growth when compared with nontransformed epithelial intestinal cells. Colonic adenocarcinoma cells contain increased numbers of lipid droplets with documented PGE_2 synthase localization and focal PGE_2 synthesis. Inhibition of lipid droplet formation by aspirin correlated with both inhibition of PGE_2 generation and cell proliferation in CACO-2 and IEC-6 H-*ras*V12 transformed cells (Accioly et al. 2008). A similar effect was obtained with lipid droplet inhibition by C75, a fatty acid synthase inhibitor. Interestingly, although C75 is not a COX inhibitor, it significantly inhibited the PGE_2 production and proliferation of colon cancer cells (Accioly et al. 2008). The inhibition of lipid body generation may affect the subcellular compartmentalization of COX-2 and, in consequence, inhibit the enhanced prostaglandin synthesis that is related to the pathogenesis of colon cancer.

Approaches to inhibit lipid accumulation in macrophage foam cells may be of therapeutic value in preventing atherosclerosis, as reviewed elsewhere (Li and Glass 2002). Different strategies to inhibit lipid droplet formation have been tested to address the role of macrophage lipid droplets as targets for therapeutic intervention

in atherosclerosis. ADRP expression facilitates foam cell formation induced by modified lipoproteins in mouse macrophages in vitro; conversely, ADRP gene inactivation in apolipoprotein E-deficient mice reduces the number of lipid droplets in foam cells that localize to atherosclerotic lesions and protects mice against the disease (Paul et al. 2008). ACAT inhibitors, including the fungal-derived cyclodepsipeptides, showed potent inhibitory activity of lipid droplet accumulation in mouse peritoneal macrophages and exerted antiatherogenic activity in both LDL receptor and apolipoprotein E knockout mice (Namatame et al. 2004).

A role for lipid droplet inhibition in host response to infection has been proposed (Bozza et al. 2009a). Accumulating evidence supports the hypothesis that lipid-droplet-derived endogenous PGE_2 downmodulates the macrophage response by inhibiting mycobacteria-induced TNF production and increasing the levels of the anti-inflammatory cytokine IL-10 (D'Avila et al. 2006, 2008). As such, pharmacological inhibition of either prostaglandin production or lipid droplet formation would have beneficial effects for the host to control the infection. Accordingly, PPARγ inhibition in macrophages not only leads to decreased lipid droplet biogenesis but also enhances macrophage mycobacterial killing, supporting the hypothesis that lipid droplets may have implications in the pathogenesis of mycobacterial infection (Almeida et al. 2009). Inhibition of lipid droplet formation or the disruption of the association of capsid viral proteins with lipid droplets lead to decreased viral particle formation in dengue infection (Samsa et al. 2009) and hepatitis C infection (Boulant et al. 2007; Miyanari et al. 2007), suggesting an effect of lipid droplets in viral replication.

The observation that some neoplastic cells exhibit increased numbers of lipid droplets has raised the possibility of using the detection of lipid droplets and/or overexpression of PAT proteins (perilipin, ADRP, and tail-interacting protein of 47 kDa) as biomarkers for specific cancer types, cancer stage, or cancer aggressiveness (Table 6.1). For example, it has been described that PAT proteins are frequently expressed in neoplastic tissues which develop some degree of steatosis, such as hepatic, colorectal, and renal cell carcinomas, albeit each of these cancer types exhibits a distinct pattern and distinct expression levels of each PAT family member (Straub et al. 2010). Among all PAT proteins, the relevance that ADRP is attaining as a potential biomarker for tumorigenesis is of particular interest. This is supported by the differential expression of ADRP in several human carcinomas such as renal cell carcinomas, hepatocellular carcinoma, colon carcinoma, and in the apocrine carcinoma of the breast (Rae et al. 2000; Kurokawa et al. 2004; Yao et al. 2005, 2007; Matsubara et al. 2011; Moritani et al. 2011). ADRP overexpression was also found in malignant or premalignant cutaneous lesions (Ostler et al. 2010). Interestingly, ADRP was recently pointed out as an interesting biomarker for Burkitt lymphoma, especially because of its specific overexpression pattern when compared with diffuse large B cell lymphomas (Ambrosio et al. 2012). Therefore, even though further work on the mechanistic impact of increased lipid body formation in tumor cells is still needed, using lipid-body-associated proteins as transformation biomarkers would be useful to distinguish tumor types and staging, as seen between two lymphomas that exhibit a similar cytological presentation.

Changes in lipid metabolism can affect numerous aspects of cell homeostasis, such as the availability of structural lipids for membrane synthesis and energy production and the magnitude of the lipid-based inflammatory mediators produced. Each of these factors has a strong impact on vital cellular processes, including cell growth, proliferation, differentiation, and motility, and thus impact on cancer promotion and growth (Santos and Schulze 2012). On the basis of this, enzymes involved in lipid mobilization from lipid storage sites such as lipid droplets may be appropriate targets for cancer therapy or for anti-inflammatory drug development. Accordingly, recent reports suggest a relationship between lipolytic and lipogenic enzymes with both tumor development and establishment of inflammatory diseases. For example, Nomura et al. (2010) showed that aggressive human cancer cells acquire the ability to liberate fatty acids from lipid neutral stores as a consequence of heightened expression of a lipolytic enzyme, monoacylglycerol lipase (MAGL). Also, this fatty acid network is enhanced by oncogenic signaling lipids, such as PGE_2 and lysophosphatidic acid, which promote migration, invasion, survival, and in vivo tumor growth (Nomura et al. 2010). Moreover, overexpression of MAGL increases the pathogenicity of nonaggressive tumor cell lines, whereas the use of a specific MAGL inhibitor was sufficient to cause this phenotype to revert (Nomura et al. 2010). Taken together, these data strongly designate MAGL as a potential pharmacological target for cancer therapy.

The discovery of a fat mobilization mechanism between stromal and cancer cells brought into the picture a possible role for lipid accumulation also in microenviroment-induced tumor growth and metastasis. Metastasis of ovarian cancer to the omentum was shown to be mediated by local adipocytes, which promoted the initial homing of tumor cells through secretion of IL-6, IL-8, MCP-1, and tissue inhibitor of metalloproteinases 1 (TIMP-1) (Nieman et al. 2011). Subsequently, adipocytes provided fatty acids to cancer cells, which displayed increased lipid droplet formation and increased activity and expression of key enzymes for β-oxidation, and consequentially developed into larger tumor masses (Nieman et al. 2011). Remarkably, this mechanism was not limited to ovarian cancer cells, but was also true for breast and colon malignant cells (Nieman et al. 2011), and provides a rationale for sustained growth of cancer metastasis targeted to an adipocyte-rich environment. Accordingly, other reports have suggested the development of specific therapies that target the cross talk and fueling of cancer metabolism by the microenvironment (Pavlides et al. 2009; Martinez-Outschoorn et al. 2010). Fatty acid binding protein 4 (FABP4), which is directly involved in the metabolic changes that occur in ovarian cancer metastasis at the omentum (Nieman et al. 2011), may be a suitable target for therapeutic intervention in a case like this. Interestingly, FABP4 was previously described in macrophages as a pivotal enzyme for cholesterol traffic and associated inflammation (Makowski et al. 2005), and an increased amount of FABP4 in plasma was also associated with carotid atherosclerosis or acute ischemic stroke (Holm et al. 2011). Impairment of FABP4 expression in macrophages suppressed the inflammatory function of these cells, including decreased expression of COX-2 and diminished PGE_2 production (Makowski et al. 2005). These findings support the relevance of lipid storage

mobilization not only for energy delivery for cancer growth, but also during formation of an inflammatory microenvironment, which could conjunctly help tumor establishment.

It has been reported that apoptosis induction leads to accumulation of lipid droplets (Callies et al. 1993; Hakumaki and Kauppinen 2000; Blankenberg 2008). Even though the mechanistic implications of this event are not known, given that the increase in the numbers of these organelles occurs very early after apoptosis induction, it is possible that lipid droplet formation may delay accumulation of toxic fatty acids. Indeed, increased lipogenesis and/or induced β-oxidation protected skeletal muscle cells and pancreatic β cells from toxicity associated with free fatty acids (Henique et al. 2010; Choi et al. 2011). In a practical application, an increase in cellular lipid droplet content has been used as an in vivo marker of posttreatment tumor cell death through ^{1}H nuclear magnetic resonance spectroscopy, a noninvasive diagnostic technique (Hakumaki et al. 1999; Blankenberg 2008). Given that lipid droplet accumulation is one of the first events that occurs on apoptosis induction, techniques based on ^{1}H nuclear magnetic resonance spectroscopy should be useful in detecting the earliest signs of cell death following cancer treatment. It was recently described that lipid accumulation in apoptotic cells is due to inhibition of mitochondrial fatty acid β-oxidation and redirection of fatty acids to de novo lipid synthesis, occurring downstream of apoptosis-induced activation of p53 and inhibition of mTOR signaling pathways (Boren and Brindle 2012). Moreover, mitochondrial dysfunction and increased levels of mitochondrial reactive oxygen species correlated with inhibition of fatty acid oxidation (Boren and Brindle 2012). Even though the relevance of lipid droplet biogenesis during apoptotic or preapoptotic events is still unknown, more evidence about the relationship between lipid droplets and cell death may attract attention to a possible function in cancer cell survival.

6.5 Concluding Remarks

Lipid droplets may influence cell functions through a variety of complex mechanisms and these mechanisms are now beginning to be unraveled. Our contemporary view of lipid droplets places these organelles as key regulators of different inflammatory and neoplastic diseases and as potential biomarkers of cell activation and diseases. Lipid droplet biogenesis is highly regulated and is cell- and stimulus-specific. Studies of the structural features of lipid droplets have revealed a much more complex structure then initially anticipated that besides lipids includes a diverse array of proteins that may differ according to the cell type and cellular activation state and thus may determine different cellular functions for this organelle. The further identification of key pathways, molecules, and functions of lipid droplets may make possible the development of therapeutic targets for future intervention in diseases that progress with increased lipid droplet accumulation as in atherosclerosis, hepatic steatosis, inflammation, and cancer. Of note, studies

targeted at the development of selective lipid droplet inhibitors will be of great interest. Moreover, further work will be necessary to determine the safety of lipid droplet inhibition since lipid accumulation within lipid droplets may participate in protective mechanisms of lipid homeostasis against cellular lipotoxicity and in early stages of apoptosis induction.

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Chapter 7
The Role of Sphingolipids in Modulating Pluripotency of Stem Cells

Salman B. Hosain, Ronald A. Hill and Yong-Yu Liu

Abstract Sphingolipids are essential components in mammalian cell membranes and vital modulators of cellular signaling processes. Nine molecular types of glycosphingolipids (GSLs), along with sphingosine 1-phosphate (S1P), play crucial roles in stem cells. GSLs are generated via ceramide glycosylation, the first step of which is catalyzed by glucosylceramide synthase, followed by further alternative elaborations to globo-series, ganglio-series, and lacto-series GSLs. Certain globo-series GSLs, namely, Gb5 (stage-specific embryonic antigen 3) and MSGb5 (stage-specific embryonic antigen 4), are essential for maintaining pluripotency of embryonic stem cells , and a shift of globo-series to ganglio-series GSLs (GD3, GM3, GD2, GT3) is associated with differentiation of embryonic stem cells into neural stem cells. Stem properties of breast cancer stem cells (BCSCs) mainly rely on glucosylceramide synthase and globo-series GSLs (Gb3). GD2 is a newly adopted surface marker for identifying BCSCs, and is also an effective target for eradicating BCSCs. S1P, which is generated from sphingosine phosphorylation following ceramide hydrolysis, activates S1P receptors and regulates stem cell proliferation and differentiation. Fingolimod deactivates S1P receptors and induces brain tumor stem cell apoptosis and protects neural stem cells. Targeting selected enzymes controlling sphingolipid synthesis and degradation can maintain normal stem cells and eradicate cancer stem cells. GSLs and S1P modulate stem cells through cSrc/β-catenin and pathways, but the mechanistic implications require further studies for clarification.

Keywords Stem cells • Glycosphingolipids • Sphingosine 1-phosphate • Extracellular-signal-regulated kinase • β-Catenin

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7.1 Introduction

Sphingolipids are ubiquitous lipid components in mammalian cell membranes, but are also intracellular signaling molecules involved in regulating many cell processes, including proliferation, apoptosis, autophagy, tumorigenesis, metastasis, and cellular responses to stress. Recent studies indicate that among sphingolipids, particularly nine molecular species of glycosphingolipids (GSLs) and sphingosine 1-phosphate (S1P) are highly associated with cell or organ development, and play important roles in regulating self-renewal and pluripotency of stem cells. We will discuss recent findings, focusing on biosynthesis of GSLs and S1P and regulation thereof; how those sphingolipids modulate the properties of embryonic stem cells (ESCs), normal adult stem cells, and cancer stem cells (CSCs); and the current progress in deciphering potential signaling pathways by which GSLs or S1P maintains the self-renewal of stem cells and determines their differentiation into other types of cells. We then suggest further studies that would help us ascertain the relative importance of particular sphingolipids in normal stem cells and CSCs, perhaps identify new approaches for inducing as well as maintaining pluripotency of normal stem cells, and for eradicating CSCs.

7.2 Biosynthesis of Sphingolipids Associated with Stem Cells

Sphingolipids play crucial roles in determining stem cell fate, including self-renewal, proliferation, and differentiation. Among the tens of thousands of sphingolipids detected (Merrill 2011), approximately nine molecular species of GSLs and S1P are highly associated with stem cells. Sphingolipids are composed of sphingoid bases linked to fatty acids (Hannun and Obeid 2008; Merrill 2011). Ceramide, a key intermediate in sphingolipid metabolism, is the simplest in structure, although in truth it is not a single molecular species, but instead represents a multiplicity of structures based on fatty acid variation in the amide moiety (see below). Other complex sphingolipids possess additional hydrophilic moieties, such as phosphate, phosphorylcholine, or sugar moieties attached to their sphingoid bases. C_{18} ceramide is the most abundant molecular species of ceramide in most mammalian cells (Fig. 7.1), although the generic term "ceramide" designates a family of more than 50 distinct molecular species (Pewzner-Jung et al. 2006; Rabionet et al. 2008). One fate of ceramide in cells is glycosylation, yielding GSLs. The simplest of such species is produced when glucose becomes attached to the 1-hydroxyl group of ceramide to form glucosylceramide (GlcCer) (Fig. 7.1). From GlcCer, more complex GSLs, such as lactosylceramide (LacCer), globotriaosylceramide (Gb3), and monosialoganglioside (GM3), can be synthesized by incorporation of additional sugar residues (Hannun and Obeid 2008; Yu et al. 2009). Hydrolysis of ceramide by ceramidase cleaves the fatty acyl chain from

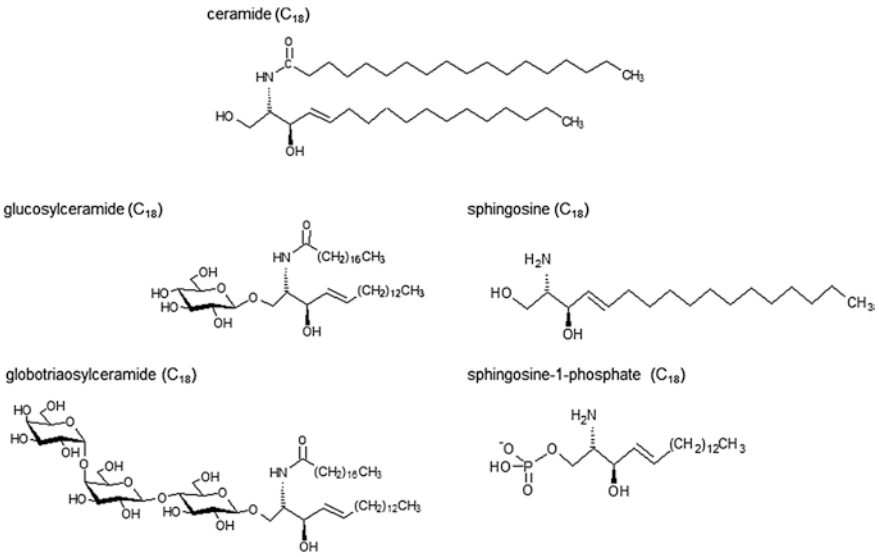

Fig. 7.1 Basic structures of ceramide, glucosylceramide, globotriaosylceramide, sphingosine, and sphingosine 1-phosphate (S1P). In mammals, the prevalent ceramide is C_{18} ceramide, which has a sphingoid base length of 18 carbon atoms, with an *E* double bond between C-4 and C-5, and a C_{18} fatty acid acylating its C-2 amino group. Glucose is attached to the 1-hydroxyl group of ceramide to form glucosylceramide. A series of glycosylations transfers galactose units to the glucose moiety of glucosylceramide to generate various glycosphingolipids (GSLs), such as globotriaosylceramide. Sphingosine (C_{18}) is the predominant sphingoid base in mammalian cells, and phosphorylation by sphingosine kinase (SphK)occurs on the hydroxyl group at C-1 of sphingosine to form S

ceramide, yielding sphingosine. Sphingosine can be phosphorylated by sphingosine kinase (SphK) to form S1P, in which phosphate is attached to the hydroxyl group of sphingosine (Fig. 7.1).

7.2.1 Synthesis of Ceramide and Its Translocation from the Endoplasmic Reticulum to the Golgi Apparatus

In mammalian cells, ceramide is synthesized predominantly by the de novo pathway, from serine and palmitoyl-CoA, in the endoplasmic reticulum (ER) and ER-associated membranes (Hannun and Obeid 2008; Merrill 2011) (Fig. 7.2). Serine–palmitoyl transferase catalyzes condensation of L-serine and palmitoyl-CoA to form 3-ketosphinganine, and the latter is quickly reduced by 3-ketosphinganine reductase to yield sphinganine (also named dihydrosphingosine) (Hanada 2003). The predominant sphinganine has 18 carbon atoms in its backbone; however, different carbon chain lengths of sphinganine (C_{12}–C_{26}) have been reported

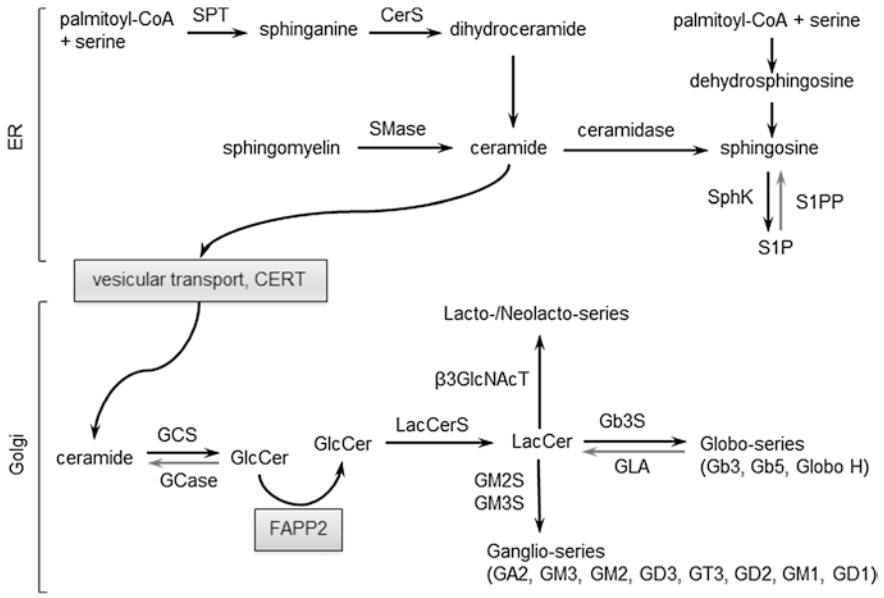

Fig. 7.2 Sphingolipid biosynthesis. Ceramide can be synthesized via the de novo pathway in the endoplasmic reticulum (*ER*), and is glycosylated to produce GSLs in the Golgi apparatus. Ceramide can also be hydrolyzed to yield sphingosine in ER. *SPT* serine–palmitoyl transferase, *CerS* ceramide synthase, *CERT* ceramide transfer protein, *GCS* glucosylceramide synthase, *LacCerS* lactosylceramide synthase, *Gb3S* globotriaosylceramide synthase, *GCase* glucocerebrosidase, *GLA* α-galactosidase A, *GM2S* GM2 synthase, *FAPP2* Golgi-associated four-phosphate adaptor protein 2, *GM3S* GM3 synthase, *S1PP* sphingosine 1-phosphate phosphatase, *SMase* sphingomyelinase

in mammalian cells from various species, and the chain lengths also vary between different tissues (Farwanah et al. 2007; Pruett et al. 2008). Sphinganine is then acylated via the action of sphinganine *N*-acyl transferase to dihydroceramide, followed by oxidation of dihydroceramide to ceramide by dihydroceramide desaturase (Guillas et al. 2003; Pewzner-Jung et al. 2006). The acylation reaction forms an amide bond, and a fatty carbon chair (C_{18}) is attached to sphinganine (C_{18}) to form ceramide (Fig. 7.1). In mammalian cells, the acylation of sphinganine is in fact conducted by a family of six enzymes, the ceramide synthases (Levy and Futerman 2010). Thus, what was at one time regarded as a single reaction turns out to be an unexpectedly complex collection of reactions, generating a diversity of ceramides with fatty acyl chains of various lengths, and in some instances with one or more double bonds or a hydroxyl group (Levy and Futerman 2010; Merrill 2011). Ceramide can also be produced from sphingomyelin breakdown catalyzed by sphingomyelinases in the inner leaflet of the plasma membrane (neutral sphingomyelinase) or the outer leaflet of the lysosomal membrane (acid sphingomyelinase) (Kolesnick et al. 1994; Hannun and Obeid 2008) (Fig. 7.2).

Cells employ two major mechanisms to mobilize ceramide: either ceramide transfer protein (CERT) or vesicular transport (Halter et al. 2007; Yamaji et al. 2008; Gault et al. 2010). CERT is a cytosolic protein that transfers ceramide from the ER to the Golgi apparatus, where it can be modified into sphingomyelin, and possibly GSLs, given that a sphingomyelin synthase and GlcCer synthase (GCS) have both been localized biochemically to the *cis*–medial Golgi network (Futerman and Pagano 1991). CERT is composed of at least three functional domains that determine its function: a pleckstrin homology (PH) domain, a FFAT domain, and a START domain, sequentially from the N-terminus to the C-terminus (Kudo et al. 2008). The PH domain is able to recognize phosphatidylinositol 4-monophosphate (PI4P) on acceptor Golgi membranes, thereby allowing directed transport to the Golgi apparatus. The FFAT domain is thought to facilitate binding to ER-resident vesicle-associated membrane protein (VAMP)-associated protein, so that CERT can only accept ceramide from the ER (Hanada et al. 2009; Derre et al. 2011). The START domain provides a hydrophobic pocket facilitating ceramide transport through the aqueous environment of the cytoplasm for delivery to the Golgi apparatus. In vitro studies have shown that phosphorylation of CERT at multiple serine residues, by casein kinase I or other kinases, results in inhibitory interaction between the START and PH domains, thus disabling PI4P binding and ceramide transfer (Kumagai et al. 2007). It is as yet unclear if such phosphorylation, or alternatively, the oligomerization that is known to occur with this protein, constitutes a general mechanism by which cellular stresses can inactivate ceramide transfer by CERT (Charruyer et al. 2008).

An alternative pathway for transport of ceramide species to the Golgi apparatus is coat-protein-dependent, and makes use of vesicular transport (Watson and Stephens 2005). The principal driving force behind the formation of vesicular carriers is the multisubunit coat protein complex (COPII). Little is known about how this pathway is regulated with respect to ceramide transport; however, vesicular transport is thought to be the major pathway delivering ceramide for use in GSL synthesis to the *cis* Golgi network (Gault et al. 2010) (Fig. 7.2).

7.2.2 Ceramide Glycosylation and GSL Synthesis

Human GCS (EC 2.4.1.80), also known as UDP-glucose:ceramide glucosyltransferase (GlcT-1), transfers glucose from uridine diphosphate glucose to ceramide, thereby producing GlcCer (Basu et al. 1968; Shukla and Radin 1990; Ichikawa et al. 1996) (Fig. 7.1). GCS, encoded by human *UGCG*, is a transmembrane protein present in the *cis* Golgi network, and has its catalytic site facing the cytosol, where newly produced GlcCer can be recognized by the four-phosphate adaptor protein FAPP2 (Fig. 7.2) (Jeckel et al. 1992; Ichikawa et al. 1996; D'Angelo et al. 2007). Unlike galactosylceramide, which is generated by galactosylceramide synthase in the ER, which functions only as a precursor for sulfatide and GM4 (Schulte and Stoffel 1993; Sprong et al. 1998), GlcCer is the precursor for more than 3,000

GSLs, the majority of all GSLs that can be produced by mammalian cells; moreover, GCS is the first rate-limiting enzyme in GSL synthesis (Radin 1994; Merrill 2011). Ceramide, the substrate for GCS, is transported by vesicles from the ER, or by CERT, and GlcCer synthesized on a cytosolic surface of the Golgi is then translocated across the Golgi membrane for higher GSL synthesis in the *trans* Golgi network (TGN) (Fig. 7.2) (D'Angelo et al. 2007; Halter et al. 2007).

FAPP2 is a cytosolic protein consisting of an N-terminal PH domain recognizing the Golgi marker PI4P, followed by a central proline-rich region, and then a glycolipid transfer protein (GLTP)-like domain toward the C-terminus (Halter et al. 2007). FAPP2 has transfer activity for GlcCer both in vitro and in cells (D'Angelo et al. 2007; Halter et al. 2007). Knocking down FAPP2 by RNA interference reduces the conversion of GlcCer to LacCer, and to downstream higher-order GSLs. It has been suggested that FAPP2 functions directly in the formation of apical carriers in the TGN. Evidence suggesting that FAPP2 regulates membrane transport from the Golgi apparatus via its glycolipid transfer function has also been brought forward. FAPP2 is a dimeric protein that has the capability to form tubules from membrane sheets (an action that is dependent on the PI4P-binding activity of the PH domain of FAPP2). FAPP2 exerts its membrane tubulating activity by binding the small GTPase Arf1 to induce membrane deformations leading to tubulation at the TGN (Cao et al. 2009). The function of the GLTP domain in FAPP2 remains unclear, but it could be involved in transferring GlcCer to the cellular site, where GlcCer can be translocated across the membrane, either at the TGN or in the ER, to function as a precursor luminally for complex GSL synthesis (D'Angelo et al. 2007; Halter et al. 2007). It is furthermore possible that FAPP2 functions as a sensor for regulating glycolipid levels in the cell. The presence of GlcCer on the cytoplasmic side of the TGN membrane could serve as a signal for FAPP2 to bind. This ensemble might then contribute to the formation and tubulation of transport carriers, which would exit from the TGN to deliver both protein and glycolipid cargos to the cell surface. A feedback mechanism would limit GlcCer translocation from the cytosolic to the luminal leaflet of the TGN when LacCer and other downstream GSLs accumulate in the luminal leaflet (Cao et al. 2009). Such a function would be in keeping with the proposition that lipid transfer proteins in general can function as biosensors regulating lipid levels in the cell (Mattjus 2009; D'Angelo et al. 2012).

In mammals, almost all of the higher-order GSLs are produced from GlcCer following additional reactions catalyzed by glycosyltransferases (Fig. 7.2) (Merrill 2011). Human LacCer synthase, also known as UDP-galactose:GlcCer β1→4-galactosyltransferase, is encoded mainly by *β4GalT-V* (GenBank accession no. AF097159) or *β4GalT-VI*. LacCer synthase transfers galactose from UDP-galactose to GlcCer to produce LacCer in the Golgi apparatus (Fig. 7.2) (Takizawa et al. 1999). LacCer is the precursor for synthesis of ganglio-series, globo-series, lacto-series, and neolacto-series GSLs (Fig. 7.1) (Sandhoff and Kolter 2003; Merrill 2011). For the ganglio-series GSLs, the enzyme responsible for the first neutral metabolite, GalNAcβ1 → 4Galβ1 → 4Glcβ1Cer (GA2,

also called asialo-GM2), is GM2 synthase (β1→4-*N*-acetylgalactosylaminyltransferase, β4GalNAcT), or GM2/GD2 synthase because it additionally converts ganglioside GM3 to GM2, ganglioside GD3 to GD2, and so forth (Furukawa and Takamiya 2002). LacCer is sialylated to ganglioside GM3 by ST3Gal-V (SAT-I, CMP-*N*-acetylneuraminate:LacCer α2→3-sialyltransferase, also known as GM3 synthase) (Yu et al. 2004). To form the disialo (GD3) and trisialo (GT3) gangliosides (NAcα2 → 8NAcα2 → 8NAcα 2 → 3Galβ1 → 4Glcβ1Cer), the additional enzymes GD3 synthase (SAT-II) and GT3 synthase (SAT-III) are involved (Merrill 2011).

Biosynthesis of Gb3 (Galα1 → 4Galβ1 → 4Glcβ1Cer), the initial step for the globo-series GSLs, is catalyzed by Gb3 synthase (Kojima et al. 2000). Gb3 is converted to Gb4 by Gb4 synthase (β3GalNAcT). Next in this series is Gb5, synthesized by the action of β3GalT-V. Biosynthesis of lacto-series and neolacto-series GSLs begins with the formation of GlcNAcβ1 → 3Galβ1 → 4Glcβ1Cer (also referred to as Lc3 or aminoceramide trihexoside) by β1→3-*N*-acetylglucosaminyltransferase (UDP-*N*-acetylglucosamine:β-galactose β1→3-*N*-acetylglucosaminyltransferase, aminoceramide trihexoside synthase) (Togayachi et al. 2001).

GSLs synthesized in the Golgi apparatus are clustered with sphingolipids and other membrane components to form GSL-enriched microdomains (GEMs), or lipid rafts, and glycosynapses found within cell membranes (Sonnino et al. 2006; Gupta and Surolia 2010; Hakomori 2010). GEMs are small, heterogeneous and dynamic domains enriched with GSLs, sphingolipids, cholesterol,and glycosylphosphatidylinositol-anchored proteins or other proteins (tetraspanins, caveolins, growth factor receptors, integrins) (Hancock 2006; Hakomori 2010). These specialized membrane microdomains profoundly influence membrane organization, and are known to compartmentalize cellular processes by serving as organizing centers for the assembly of signaling molecules, influencing membrane fluidity and membrane protein trafficking, and regulating neurotransmission and receptor trafficking (Simons and Ikonen 1997; Lingwood and Simons 2010). GEMs modulate membrane transport, signal transduction, and cell–cell interactions, among other functions (Lingwood and Simons 2010; Liu et al. 2010).

7.2.3 Synthesis of Sphingosine and Sphingosine 1-Phosphate

Sphingoid bases consist mostly of 18 carbons in mammalian sphingolipids, primarily because of the preference of serine–palmitoyl transferase for saturated fatty acyl-CoAs and the abundance of palmitoyl-CoA in mammalian cells (Merrill and Williams 1984; Haynes et al. 2008; Pruett et al. 2008). Sphingosine is an 18-carbon amino alcohol (2-amino-4-octadecene-1,3-diol) with a monounsaturated hydrocarbon chain, which forms a primary part of sphingolipids (Fig. 7.1). Sphingosine can be generated via ceramide hydrolysis, in which ceramidase

deacylates ceramide, yielding sphingosine and a free fatty acid (Fig. 7.2). Five human ceramidases have been found: acid ceramidase, neutral ceramidase, and alkaline ceramidases (ACER1, ACER2, ACER3) (Nikolova-Karakashian and Merrill 2000; Mao and Obeid 2008). The various ceramidases are distributed in different compartments of cells, neutral ceramidase appearing predominantly in the plasma membrane (Tani et al. 2003; Hwang et al. 2005), acid ceramidase in the lysosomal membrane (Bernardo et al. 1995), and alkaline ceramidases in the ER/Golgi complex (Mao et al. 2001; Mao et al. 2003; Xu et al. 2006).

Sphingosine can be further phosphorylated by SphK, yielding S1P (Fig. 7.2) (Olivera and Spiegel 1993). There are two isoforms of SphK in mammalian cells, $SphK_1$ and $SphK_2$. $SphK_1$ is encoded by the *SPHK1* gene, and is mainly localized in the cytosol, but this enzyme can be recruited to membranes rich in phosphatidate, a product produced primarily by the action of phospholipase D (Pitson 2011). In contrast to $SphK_1$, $SphK_2$ is present in several intracellular compartments, including the cytosol and the nucleus, with its distribution being dependent on the cell type (Igarashi et al. 2003). Most of the known actions of S1P are mediated by a family of five specific G-protein-coupled receptors ($S1PR_1$–$S1PR_5$) (Pyne and Pyne 2010). $SphK_2$ in the nucleus regulates gene transcription, at least in part by producing S1P, which acts as an endogenous inhibitor of histone deacetylases (Hait et al. 2009). Microsomal S1P phosphatase degrades S1P, thereby modulating S1P as a first and second messenger (Sciorra and Morris 2002). S1P is also irreversibly catabolized by S1P lyase, a highly conserved enzyme that catalyzes the cleavage of S1P at the C-2–C-3 bond, resulting in the formation of hexadecenal and ethanolamine phosphate (Pyne and Pyne 2010).

7.3 Sphingolipids and Stem Cells

Among sphingolipids, particularly the GSL species [Gb3, Gb5, monosialosyl Gb5 (MSGb5), globo H, GM1, GM3, GT3, GD2, GD3] and S1P are considered to be crucial molecules in stem cells, on the basis of a relatively large and growing body of evidence. Gb5 and MSGb5 have been found to be associated with pluripotency of ESCs, and on that basis have often been used to identify human ESCs for nearly 20 years (Thomson et al. 1995). These sphingolipids are now known to act as extracellular as well as intracellular signaling molecules, maintaining pluripotency and regulating proliferation and differentiation of ESCs, adult stem cells, and CSCs (Tables 7.1, 7.2, 7.3).

7.3.1 GSLs and Stem Cells

The cell-surface globo-series GSLs, Gb5 and MSGb5, are also known as stage-specific embryonic antigen 3 (SSEA-3) and stage-specific embryonic

Table 7.1 Sphingolipids and stem cells

Stem cells	Sphingolipids	Methods	Inducers	References
ESCs				
Human H1, H7	SSEA-3	ICC, FCM	NA	Thomson et al.(1998),
Human H9, H13, H14	SSEA-4			Draper et al. (2002)
Human HES5, H9	Gb5, Glob H, Gb4, MSGb5	MS/MS ICC	NA	Liang et al. (2010)
Human ESCs	Gb5, Globo H Gb4, MSGb5	MS/MS	NA	Liang et al. (2011)
H1, H9, HES3, CA2	SSEA-3, A2B5	FCM, ICC	NA	Hong et al. (2011)
Mouse E14	GM3, GM1, GD1a, LacCer, Gb3, Gb4	TLC	NA	Kimber et al. (1993)
Mouse TC-1	GlcCer, LacCer	TLC	NA	Yamashita et al. (1999)
Mouse J1	GM1, GM3, GD3	TLC	NA	Kwak et al. (2006) Jung et al. (2009)
iPSCs from dermal fibroblasts	SSEA-3, SSEA-4	ICC	Oct3, Sox2, Klf, c-Myc	Takahashi et al. (2007)
IMR90-3 iPSCs	SSEA-3, SSEA-4	FCM	Oct4, Sox2, Nano2, Lin28	Yu et al. (2007)
Human HES2–HES4	S1P	Treatment	PDGF	Pebay et al.(2005)
Human Shef 1–6	S1P	Treatment		Inniss and Moore (2006)
Mouse ES-D3, CGR8	S1P	Treatment		Rodgers et al. (2009)
Neural stem cells				
Human	GD3, GM3	MS/MS, FCM	NA	Liang et al. (2011)
	A2B5	FCM, ICC	NA	Pruszak et al. (2007)
	GD2	FCM	NA	Klassen et al. (2001)
	SSEA-4	ICC, FCM	NA	Barraud et al. (2007)
Neurogenesis	S1P	KO mouse	NA	Mizugishi et al. (2005)
Mesenchymal stem cells				

(continued)

Table 7.1 (continued)

Stem cells	Sphingolipids	Methods	Inducers	References
Human	GM1	TLC, ICC	NA	Kwak et al. (2006)
	GD2	ICC, FCM	NA	Martinez et al. (2007)
	GD2	ICC, FCM	NA	Xu et al. (2009)
	GM3	TLC	NA	Kim et al. (2008)
	SSEA-4	FCM	NA	Gang et al. (2007)
Hematopoietic stem cells	Gb3	TLC	NA	Cooling et al. (2003)
	S1P	FCM	NA	Golan et al. (2012)
Mesoangioblasts	S1P	Treatment	NA	Donati et al. (2007)
Muscle stem cells (satellite cells)	S1P	Treatment		Saba and de la Garza-Rodea (2013)
Human liver stem cells	SSEA-4	ICC	NA	Dan et al. (2006)

A2B5, antibody against ganglioside GT3, *ESCs* embryonic stem cells, *FCM* flow cytometry, *GlcCer* glucosylceramide, *ICC* immunocytochemistry, *iPSCs* induced pluripotent stem cells, *KO* knockout, *LacCer* lactosylceramide, *MSGb5* monosialosyl Gb5, *MS/MS* tandem mass spectrometry, *NA* not applicable, *PDGF* platelet-derived growth factor, *S1P* sphingosine 1-phosphate, *SSEA* stage-specific embryonic antigen, *TLC* thin-layer chromatography

Table 7.2 Sphingolipids and cancer stem cells

Cancer stem cells	Sphingolipids	Methods	Inducers	References
Glioblastoma	A2B5	FCM, ICC	NA	Ogden et al. (2008)
Oligodendroglioma	A2B5	FCM, ICC	NA	Tchoghandjian et al. (2010)
	S1P	Treatment	Fingolimod	Estrada-Bernal et al. (2012)
Breast cancer	Gb3, GlcCer	TLC	Doxorubicin	Bhinge et al. (2012)
	GD2	FCM	NA	Battula et al. (2012)
	SSEA-3, Globo H	FCM	NA	Chang et al. (2008)

Table 7.3 Agents targeting sphingolipids associated with human stem cells

Agents	Target	Activity	Stem cells	References
Ceramide	SphK, ceramidases	Induction	Human ESCs	Salli et al. (2009)
Doxorubicin	GCS	Induction	Breast CSCs	Bhinge et al. (2012)
Fingolimod	S1PRs	Inhibition	Brain CSCs	Estrada-Bernal et al. (2012)
MBO-asGCS	GCS	Inhibition	Breast CSCs	Bhinge et al. (2012)
Triptolide	GD3S	Inhibition	Breast CSCs	Battula et al. (2012)
shRNA	GD3S	Inhibition	Breast CSCs	Battula et al. (2012)

CSCs cancer stem cells, *GCS* glucosylceramide synthase, *GD3S* GD3 synthase, *MBO-asGCS* antisense mixed-backbone oligonucleotide targeted against glucosylceramide synthase, *shRNA* short hairpin RNA, *SphK* sphingosine kinase, *S1PRs* sphingosine 1-phosphate receptors

antigen 4 (SSEA-4), and are frequently relied on as markers of undifferentiated human primary ESCs, as well as derived or induced ESCs (Kannagi et al. 1983; Thomson et al. 1995, 1998; Richards et al. 2002; Klimanskaya et al. 2005, 2006; Liang et al. 2010). SSEA-3 and SSEA-4 are highly expressed in many human ESC lines (H1, H7, H9, H13, H14, HES3, CA2, HES5) and in isolated human ESCs (Table 7.1) (Thomson et al. 1998; Draper et al. 2002; Liang et al. 2010, 2011), and their levels are downregulated after differentiation (Draper et al. 2002; Liang et al. 2010). Human induced pluripotent stem cells generated from fibroblasts by transduction of Oct3, Sox2, Klf4, and c-Myc genes (or Oct3, Sox2, Nano2, Lin28) were positive for SSEA-3 and SSEA-4 (Takahashi et al. 2007; Yu et al. 2007). In contrast to human ESCs, mouse ESCs were found to have higher levels of GM1, GM3, GD3, and LacCer (Kimber et al. 1993; Yamashita et al. 1999; Kwak et al. 2006; Jung et al. 2009). With tandem mass spectrometry, flow cytometry, and other methods, Liang et al. (2010) found that globo-series and lacto-series GSLs prominently present were replaced with ganglio-series GSLs on ESC differentiation to an embryoid body having three germ layers. In addition to SSEA-3 and SSEA-4, several globo-series and lacto-series GSLs, including Gb4, LacCer, fucosyl LacCer, Globo H, and disialyl Gb5, were also found to be present in human ESCs (HES5 and H9); however, during differentiation into embryoid bodies elicited by depletion of basic fibroblast growth factor (bFGF) in the culture medium, mass spectrometry revealed a clear-cut switch in the glycosylation patterns of the prominent GSLs, globo series and

lacto series to ganglio series, owing to the upregulation of the GM2 synthase and GM3 synthase catalyzing ganglio-series GSL biosynthesis, and simultaneous downregulation of Gb3 synthase and β3GlcNAcT needed for synthesis of ganglio-series GSLs (Liang et al. 2010). The alterations in GSL patterns were further clarified as being correlated with lineage-specific differentiation of ESCs. During differentiation of human ESCs into neural progenitors, the glycosylation of prominent GSLs switched from globo series and lacto series to mostly ganglio series, in this case dominated by GD3 (Liang et al. 2011). On the other hand, when ESCs differentiated into endodermal cells, the most prominent GSL was Gb4 (Liang et al. 2011). Several studies showed that neural stem cells have higher levels of GD3, GM3, GT3, and MSGb5 (Klassen et al. 2001; Barraud et al. 2007; Pruszak et al. 2007; Liang et al. 2011). A2B5, a monoclonal antibody binding to the c-series ganglioside GT3 or its 9-O-acetylated derivative (Dubois et al. 1990), was used as a cell-sorting marker isolating neural stem cells (Pruszak et al. 2007). Ganglio-series GSLs, including GD2, GM1, GD2, GM3, and SSEA-4, were prominent in mesenchymal stem cells (Kwak et al. 2006; Gang et al. 2007; Martinez et al. 2007; Kim et al. 2008). GD2 and SSEA-4 were used as markers for separation of mesenchymal stem cells (Gang et al. 2007; Martinez et al. 2007). SSEA-4 was found in multipotent progenitors isolated from human fetal liver (Dan et al. 2006). Galactosylceramidase catalyzes hydrolytic removal of galactose from galactosylceramide or galactosylsphingosine, yielding ceramide or sphingosine, respectively. It was reported that galactosylceramidase was involved in maintaining a functional hematopoietic stem cell niche by control of the intracellular ceramide and sphingosine levels (Visigalli et al. 2010).

Direct evidence of GSLs modulating stem cell character comes from the study of a GCS-knockout mouse (Yamashita et al. 1999). In the absence of GSL synthesis, ESCs were able to differentiate into endodermal, mesodermal, and ectodermal derivatives, but were deficient in their ability to form well-differentiated tissues, because of which embryos died at day E7.5 (Yamashita et al. 1999). Human dental-pulp-derived stem cells normally have the potential to differentiate into multiple cell lineages; however, knockout of GCS with small interfering RNA prevented their differentiation into neural cells (Ryu et al. 2009). Human ESCs that expressed higher levels of ceramide-metabolizing enzymes (acid ceramidase, $SphK_1$, $SphK_2$, galactosylceramide synthase, ceramide kinase) capable of converting ceramide into promitogenic metabolites were resistant to the normally lethal ceramide, and propagated in medium with added nanoliposomal ceramide, whereas ceramide selectively eliminated cells that differentiated into neuroectoderm with nestin and Tuj1 markers (Salli et al. 2009). Treatment with C_2 ceramide activated protein phosphatase 2A (PP2A), leading to differentiation of human ESCs (HSF-6 and Miz-hES4), whereas okadaic acid (a PP2A inhibitor) or bFGF suppressed PP2A, facilitating sustained ESC self-renewal through signaling pathways mediated by glycogen synthase kinase 3β (Yoon et al. 2010). Stem cells may protect themselves by

converting ceramide to GlcCer and other GSLs; thus, ceramide glycosylation becomes a turning point for maintenance versus death or differentiation of stem cells (Bieberich 2004). Silencing of GCS expression with short hairpin RNAs decreased the levels of GM3 and GD3 in mouse ESCs, inhibited ESC proliferation, and diminished the potential of mouse ESCs to differentiate into glial cells and neurons (Jung et al. 2009). This same study further showed that GSLs possibly activated the extracellular-signal-regulated kinase (ERK) 1/2 pathway to modulate ESC proliferation (Jung et al. 2009). Neuroepithelial cells are the "stem cells" of the nervous system, and can differentiate into neural and glial cells during neural development. Inhibition of ceramide glycosylation with the GCS inhibitor D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol depleted GD3, a major ganglioside of neuroepithelial cells, in GEMs and repressed bFGF-induced proliferation of neuroepithelial cells mediated via the Ras–mitogen-activated protein kinase (MAPK) pathway (Yanagisawa et al. 2005). This study suggests that GSLs are directly involved in regulating the proliferation of neural stem cells.

Interestingly, particular GSL species and the enzymes for their syntheses, which according to the weight of evidence act as modulators of stem cell fate, have been found to be associated with CSCs (Tables 7.2, 7.3). SSEA-3 and globo H are highly expressed in certain types of stem cells present in breast cancers (Chang et al. 2008). A2B5 was used as a surface marker, along with CD133, to identify CSCs ($A2B5^+/CD133^-$) in adult human gliomas (Ogden et al. 2008; Tchoghandjian et al. 2010). GCS was seen to be overexpressed in breast CSCs (BCSCs; $CD24^-/CD44^+$/epithelial specific antigen positive), and levels of the globo-series GSLs, particularly Gb3, were significantly higher in BCSCs than in other differentiated cancer cells. Furthermore, silencing of GCS expression with a suitable antisense mixed-backbone oligonucleotide (MBO-asGCS) decreased BCSC numbers (Gupta et al. 2011; Bhinge et al. 2012). In contrast, the levels of GCS and of GSLs were lower in bone marrow stem cells, and bone marrow stem cells were not sensitive to GCS suppression (Bhinge et al. 2012). More recently, Battula et al. (2012) reported that ganglioside GD2 can be used to identify CSCs. $GD2^+$ cells, which have been detected in 12 different breast cancer cell lines and in patient samples (12 cases), are capable of forming mammospheres and initiating tumors with as few as ten $GD2^+$ cells (Battula et al. 2012). In addition, the majority of $GD2^+$ cells are also $CD44^{hi}/CD24^{lo}$, the previously established CSC-associated cell surface phenotype. Gene expression analysis revealed that the enzyme GD3 synthase is highly expressed in $GD2^+$ as well as in $CD44^{hi}/CD24^{lo}$ cells; interference with GD3 synthase activity, either by short hairpin RNA or by an inhibitor, triptolide, reduced the CSC population and inhibited CSC-associated properties. Furthermore, GD3 synthase knockdown completely abrogated tumor formation in vivo. Lately, induction of epithelial–mesenchymal transition in transformed human mammary epithelial cells (HMLER cells) dramatically increased GD3 synthase expression and the GD2 level (Battula et al. 2012).

7.3.2 Sphingosine 1-Phosphate and Stem Cells

S1P is generated by SphKs and released via ATP-binding cassette transporters mainly from platelets, erythrocytes, and vascular endothelial cells. Extracellular S1P generates intracellular signals through its specific cell agonistic actions on surface receptors (namely, $S1PR_1$–$S1PR_5$), which are G protein-coupled receptors (Pyne and Pyne 2010; Maceyka et al. 2012). S1P plays a role in regulating fate in various stem cell types (Pitson and Pebay 2009) (Table 7.1). Human ESCs are target cells of S1P signaling, as various human ESC lines, including Shef 1-6 and HES2–HES4, express $S1PR_1$–$S1PR_5$ (Pebay et al. 2005; Inniss and Moore 2006). Human induced pluripotent stem cells (hiPS1) were found to express the messenger RNAs for $S1PR_1$ and $S1PR_3$ (Pitson and Pebay 2009). S1P has proven to be a valuable component of a defined culture medium for maintaining human ESCs. In serum-free medium with mouse embryonic fibroblasts, S1P (20 μM) reduced apoptosis in human ESC lines Shef 1-6 while increasing their proliferation (Inniss and Moore 2006). S1P alone did not substantially prevent spontaneous differentiation of human ESCs; however, co-incubation of S1P (10 μM) with platelet-derived growth factor (PDGF; 20 ng/ml) allowed long-term maintenance of human ESC lines HES2, HES3, and HES 4 in an undifferentiated state in a serum-free medium (Pebay et al. 2005). S1P displayed antiapoptotic effects associated with activation of ERK1/2 signaling, but not involving activation of Akt, even though PDGF stimulated both ERK1/2 and Akt (Wong et al. 2007). Microarray analysis showed S1P to be important to human ESC status and population maintenance, as it upregulated antiapoptotic, cell cycle progression, and cell adhesion genes, while downregulating proapoptotic genes in human Shef 4 ESCs; S1P also downregulated pluripotency genes, in particular *nanog* and *Oct-4* (Avery et al. 2008). Mouse ESCs also express S1PRs. Cells of mouse ESC line R1 expressed $S1PR_1$–S1PR5; however, $S1PR_4$ was not found in mouse CGR8 and ES-D3 cells (Kleger et al. 2007; Rodgers et al. 2009). S1P is a stimulatory regulator of mouse ESC proliferation, as S1P activated $S1PR_5$ and thereby ERK1/2 signaling, via mechanisms dependent on G_i, protein kinase C, and cSrc, to increase cell proliferation (Rodgers et al. 2009).

S1P modulates multipotency maintenance, differentiation, and motility of various adult stem cells. Mesoangioblasts are stem cells capable of differentiating into various mesodermal tissues, such as smooth and striated muscle, bone, and endothelium. S1P acted as a potent mitogen and antiapoptotic agent in murine (D16 clone) and human mesoangioblasts (Donati et al. 2007). S1P (1 μM, 24 h) promoted proliferation of mesoangioblasts via $S1PR_2$ activation. On the other hand, S1PRs were not implicated in the antiapoptotic effect of S1P (Donati et al. 2007). The antiapoptotic effects of transforming growth factor β on mesoangioblasts involved the activation of $SphK_1$, causing increased S1P formation (Donati et al. 2009). S1P in turn upregulated the transcription factors GATA6 and LMCD1 as well as smooth muscle contractile proteins, and triggered differentiation of mesoangioblasts toward the smooth muscle phenotype (Donati et al. 2011). S1P serves

as a muscle trophic factor that facilitates efficient muscle regeneration, due in part to the ability of S1P to activate quiescent muscle stem cells, called satellite cells, which are needed for muscle repair. S1P activated quiescent satellite cells via a pathway dependent on $S1PR_2$/signal transducer and activator of transcription 3 (STAT-3), thereby facilitating skeletal muscle regeneration (Saba and de la Garza-Rodea 2013).

S1P and S1PRs mediate homing and trafficking of progenitor cells. A S1P concentration gradient between bone marrow and blood and the presence of $S1PR_1$ on hematopoietic stem cells regulate progenitor cell egress and mobilization (Golan et al. 2012; Juarez et al. 2012; Ratajczak et al. 2012). In vivo desensitization of S1PRs by fingolimod (also named FTY720) (Gilenya) reduced steady-state egress of immature progenitors and primitive Sca-1^+/c-Kit^+/Lin^- cells via inhibition of release of stromal-cell-derived factor 1 (SDF-1). Administration of granulocyte colony-stimulating factor to mice with deficiencies in either S1P production or $S1PR_1$ expression resulted in reduced stem/progenitor cell mobilization, even though granulocyte colony-stimulating factor increased S1P levels and elevated the populations of these immature cells in mice expressing surface $S1PR_1$ (Golan et al. 2012). S1P, through its activation of the Wnt/BMP signaling pathway, was involved in osteoclast recruitment of osteoprogenitors to the site of bone remodeling (Pederson et al. 2008). Either S1P or fingolimod stimulated migration and proliferation of endothelial progenitor cells and bone-marrow-derived mononuclear cells in neovascularization, via activation of $S1PR_3$ (Walter et al. 2007). Mechanistically, S1P was found in this study to induce phosphorylation of CXCR4, activate Src kinase, and stimulate phosphorylation of JAK2.

S1P signaling plays crucial roles in CSCs, particularly brain tumor stem cells (Tables 7.2, 7.3). High expression levels of $SphK_1$ correlated with a threefold shorter average survival of glioblastoma patients (Van Brocklyn et al. 2005). Neural precursor cells derived from ESCs, and which express $S1PR_1$, can be induced by S1P or fingolimod to differentiate into an oligodendroglial lineage; however, residual pluripotent stem cells pose a risk of forming tumors in transplantation, owing to a lack of $S1PR_1$ on residual pluripotent stem cells (Bieberich 2011). $SphK_1$ and S1P drove the survival, proliferation, and invasion of primary glioblastoma brain tumor stem cells in the tumor spheres (Estrada-Bernal et al. 2011); in contrast, fingolimod treatment of brain tumor stem cells inactivated ERK, leading to upregulation of the BH3-only protein Bim, and thereby apoptosis (Estrada-Bernal et al. 2012). Fingolimod also slowed growth of intracranial xenograft tumors in nude mice and augmented the therapeutic effects of temozolomide in treating glioblastoma. Fingolimod (10 nM) was furthermore capable of increasing the viability and neurogenicity of irradiated neural stem cells from the hippocampus by promoting the survival and differentiation of neural progenitors. In contrast, this drug did not provide radioprotection in human MCF-7 breast cancer cells, nor in U-87 and C6 glioma cell lines (Stessin et al. 2012). Fingolimod is quite lipophilic and is known to cross the blood–brain barrier. This

drug recently received FDA approval for treatment of relapsing multiple sclerosis. Thus, fingolimod may potentially be an excellent therapeutic agent for treatment of glioblastoma.

7.4 Signaling Pathways Underlying Sphingolipids Modulate Stem Cells

The findings discussed so far and summarized in Tables 7.1, 7.2, and 7.3 indicate that sphingolipids modulate stem cells with respect to pluripotency maintenance, proliferation, and differentiation, and also affect tumorigenesis and cancer progression via CSCs. In general, GSLs are highly associated with pluripotency maintenance of stem cells and progenitors, and S1P is associated with regulation of differentiation. Particular species of GSLs, or particular S1PR isoforms that are normally expressed in ESCs or other early-stage stem cells, are also found to be strongly associated with tumorous potency of CSCs. Two strategies are proposed to more fully elucidate how GSLs (Fig. 7.3) and S1P (Fig. 7.4) are involved in the regulation of normal stem cells and CSCs; however, these should be further explored in more extensive systematic studies.

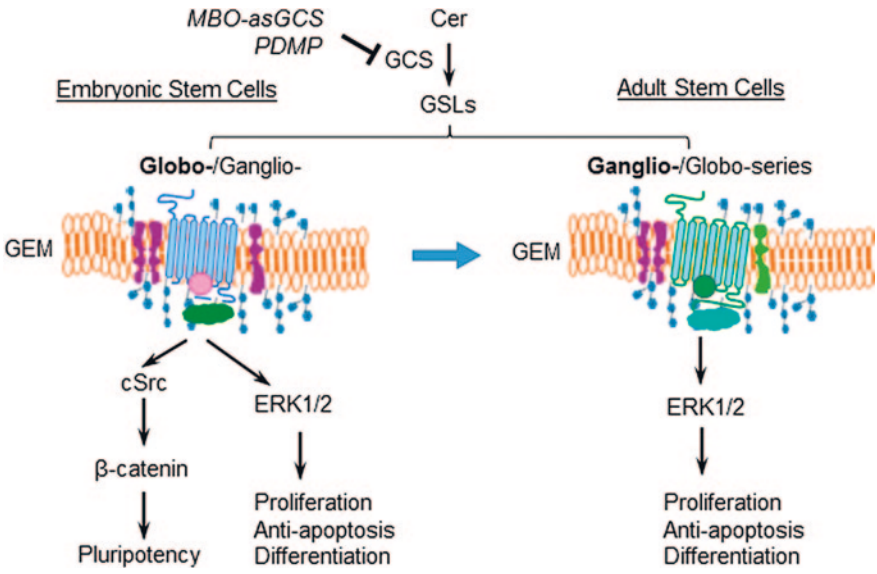

Fig. 7.3 GSLs modulate stem cell properties by GSL-enriched microdomain (*GEM*)-mediated cSrc/β-catenin and extracellular-signal-regulated kinase 1/2 (*ERK1/2*) signaling pathways. *Cer* ceramide, *MBO-asGCS* mixed-backbone oligonucleotide targeted against glucosylceramide synthase, *PDMP* D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol

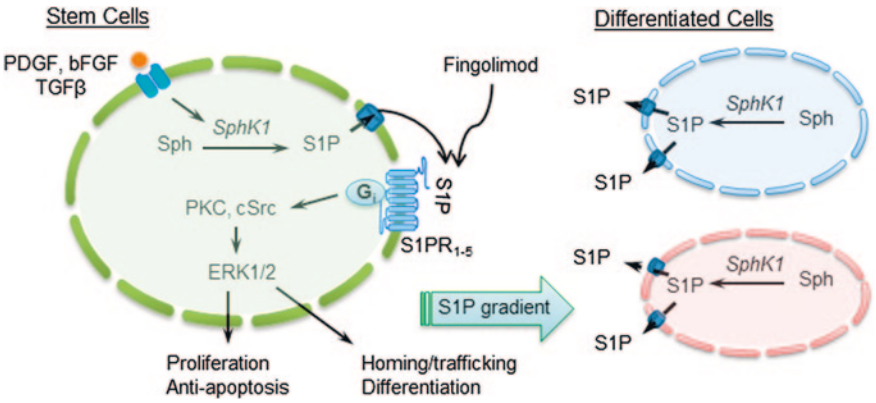

Fig. 7.4 S1P regulates stem cells in proliferation, differentiation, and homing/trafficking by S1P receptor (*S1PR*)-mediated activation of the ERK1/2 signaling pathway. *bFGF* basic fibroblast growth factor, *PDGF* platelet-derived growth factor, *TGFβ* transforming growth factor β

7.4.1 GSLs Modulate Stem Cells: GEM-Mediated ERK1/2 and cSrc/β-Catenin Signaling Pathways

GSLs are integrated with other molecules, including a number of protein kinases in GEMs of a cell membrane, and alterations of GSLs can significantly affect cellular signaling and modulate cell functions (Hancock 2006; Hakomori 2010). As previously discussed (and see Fig. 7.3), GSLs possibly activate the ERK1/2 pathway mediated through Ras–MAPK within GEMs, to modulate ESC proliferation (Yanagisawa et al. 2005; Jung et al. 2009). One of our recent studies shows that overexpression of GCS maintains pluripotency of CSCs via Gb3, with involvement of cSrc- and β-catenin-associated pathways (unpublished data). Higher levels of Gb3 and other globo-series GSLs were found in BCSCs than in cells of other subpopulations by tandem mass spectrometry. Conversely, suppression of these GSLs by silencing of GCS diminished BCSC numbers. Furthermore, studies of the effects of cSrc and β-catenin inhibition indicated that Gb3 can activate cSrc/β-catenin pathways to upregulate bFGF and Oct4 in stem cells (unpublished data). Suppressing PP2A, which as noted in Sect. 7.3.1 can be activated by ceramide, maintains human ESC self-renewal through signaling pathways mediated by glycogen synthase kinase 3β (Yoon et al. 2010). It is possible that increased levels of GSLs, due to enhanced ceramide glycosylation with concomitant decreases in ceramide levels, activated cSrc- and β-catenin-mediated signaling to maintain stem cell properties. It is still unclear, however, which species of GSLs and what associated signaling pathways regulate asymmetric and symmetric proliferation of stem cells. It is believed that the division polarity (symmetric or asymmetric) is particularly important with respect to stem cell fate decisions, and for maintenance of stem cell adhesion and quiescence (interaction within the niche) (Levy and Futerman 2010).

7.4.2 S1P Modulates Stem Cells: S1PR-Mediated Activation of the ERK1/2 Signaling Pathway

S1P generated from stem cells and other cells activates $S1PR_1$–$S1PR_5$, and thereby, signaling pathways involving protein kinase C, cSrc, and ERK1/2 in stem cells (Fig. 7.4). Other growth factors, including PDGF, bFGF, and transforming growth factor β, can activate their receptors on the plasma membrane, and thereby enhance $SphK_1$ activity, in turn increasing cellular levels of S1P. S1P-stimulated signaling generally favors stem cell proliferation and avoidance of apoptosis. As discussed in Sect. 7.3.2, the S1P gradient between stem cells and differentiated cells (neurons, blood cells) facilitates stem cell homing/trafficking, and concomitantly governs differentiation into particular types of cells. It is, however, not yet clear exactly how the S1P –ERK1/2 signaling pathways modulate symmetric versus asymmetric proliferation of stem cells or progenitors. Moreover, failure to properly regulate stem cell polarity might result in tissue atrophy or various other diseases (Levy and Futerman 2010).

7.5 Perspectives

Compared with the study of protein functions in stem cells, it remains a great challenge to identify the roles of particular species of sphingolipids in modulating stem cells owing to lack of specific ligands and inhibitors, the difficulty in delivering lipids to cells, the vast multiplicity of potential molecular species that may be present in low (yet not inconsequentially so) levels and in multiple intracellular membranes as well as the plasma membrane, and the general difficulties of characterizing and assessing the effects of dynamic changes of sphingolipids via multiple routes in metabolism. As summarized in Table 7.3, several agents have been demonstrated to affect stem cells with respect to maintaining pluripotency or altering proliferation and differentiation. Although sphingolipids are clearly involved in regulating stem cell pluripotency, much remains to be understood. The prominent stature of globo-series GSLs, such as Gb3, detailed earlier in this chapter, suggests that particular emphasis be given to investigating this class of GSLs. A more complete understanding of how various sphingolipid species selectively modulate asymmetric proliferation of stem cells should reveal new approaches to exploiting the presence of stem cells in vivo for therapeutic benefits. On the other hand, investigating molecular mechanisms by which particular species, such as Gb3 and GD2, are reexpressed in CSCs will likely provide us with alternative targets for eradicating CSCs, and therefore improving cancer treatments.

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Part II
Signaling Pathways Underlying Tumor Genesis, Proliferation and Cell Death

Chapter 8
Signaling Pathways of Cbl-b and Its Role in Peripheral T Cell Tolerance

K. Venuprasad

Abstract The E3 ubiquitin ligase Cbl-b acts as a key regulator of T cell activation and peripheral tolerance. Cbl-b deficiency uncouples the requirement of CD28 costimulation for T cell activation and exhibits resistance to T cell anergy and tolerance. Cbl-b$^{-/-}$ mice are highly susceptible to autoimmune diseases. Conversely, they mount a robust antitumor response resulting in spontaneous tumor rejection. Therefore, modulation of Cbl-b might provide a unique opportunity for immunotherapeutic strategies for inflammatory disorders, including autoimmunity and cancer.

Keywords Ubiquitination • Cbl-b • Tolerance • Anergy • Regulatory T cells

8.1 Introduction

Ubiquitin conjugation is a highly ordered multistep enzymatic process accomplished by the formation of an isopeptide bond between the C-terminal carboxyl group of ubiquitin and the α-amino group of a lysine residue of the protein substrate (Hershko and Ciechanover 1998). Among the diverse functions regulated by the ubiquitin system, the modulation of the immune response is one of the most interesting and complex (Venuprasad et al. 2006). Ubiquitination modulates diverse pathways involved in T cell activation, differentiation, and tolerance.

Ubiquitination occurs through the sequential action of at least three enzymes (Pickart 2001). Ubiquitin is first activated by a ubiquitin-activating enzyme or E1 and this forms an active thioester bond between the C-terminal glycine residue of ubiquitin and a cysteine group of E1. Activated ubiquitin is then transferred to ubiquitin-conjugating enzymes (E2s). The E3 ubiquitin ligases (E3s) are required for the covalent isopeptide bond formation between ubiquitin and the ε-amino group of lysine

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residues in the substrate protein. E3s recruit both ubiquitin-loaded E2s and the target protein, and they facilitate the transfer of ubiquitin from E2 to the substrate. Thus, the E3s are the critical components of the ubiquitin conjugation machinery and provide specificity to the ubiquitin conjugation system by direct and specific interaction with the substrate (Pickart 2001). The importance of ubiquitin in immune response is highlighted in vivo by the observation that their genetic alteration, abnormal expression, or dysfunction is accompanied by inflammatory disorders, including autoimmunity and cancer (Hershko and Ciechanover 1998; Venuprasad 2010).

8.2 Casitas B-lineage Lymphoma Family of E3 Ligases

E3 ligases are broadly classified into two families based on their catalytic motifs: proteins that contain a homologous to the E6-AP carboxy terminus (HECT) domain (HECT type) and proteins that contain a really interesting new gene (RING) finger domain (RING type) (Liu 2004). The Casitas B-lineage lymphoma (Cbl) proteins are encoded by a highly conserved gene family belonging to the RING-type E3 ligases (Loeser and Penninger 2007a). The name is derived from murine Cas NS-1 retroviral oncoprotein v-Cbl, which promotes B-linage lymphoma development in mice. Mammals express three members, c-Cbl, Cbl-b, and Cbl-3 (Loeser and Penninger 2007a). c-Cbl is overexpressed in the thymus, whereas Cbl-b is predominantly expressed in peripheral lymphoid organs (Loeser and Penninger 2007b).

All Cbl proteins contain a four-helix bundle, a calcium binding EF-hand, and a variant Src homology 2 domain, which together form the tyrosine kinase binding (TKB) domain (Loeser and Penninger 2007a). The TKB domain is followed by a helix linker region and the RING domain, responsible for its E3 ligase catalytic function (Meng et al. 1999; Nau and Lipkowitz 2003) (Fig. 8.1). Additionally, c-Cbl and Cbl-b contain proline-rich stretches that mediate the association with Src homology 3 domain containing proteins, serine and tyrosine phosphorylation sites, and a ubiquitin-associated/leucine zipper domain required for dimerization (Nau and Lipkowitz 2003; Davies et al. 2004). The TKB domain is required for specific binding to phosphorylated tyrosine residues present on activated receptors or kinases (Loeser and Penninger 2007a). Cbl proteins not only bind and regulate phosphorylated target proteins, they also themselves harbor phosphorylation sites that facilitate substrate binding.

8.3 Phosphorylation-Dependent Activation of Cbl-b

The regulation of ligase function of Cbl-b is not clearly understood. It was shown that the activity of Cbl-b is upregulated by the phosphorylation of Y363 (Y371 in c-Cbl), which is located in the helix linker region (Thien et al. 2001;

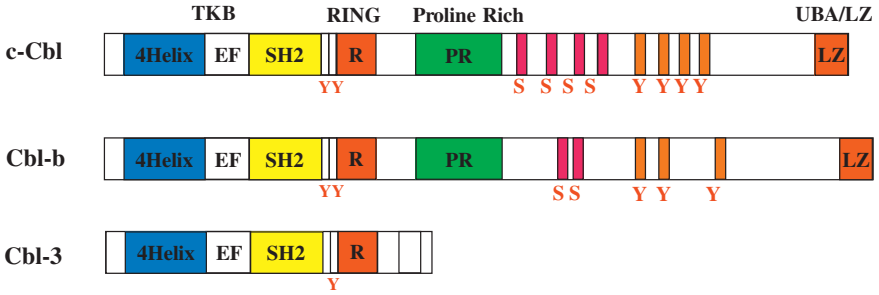

Fig. 8.1 The domain structure of mammalian Cbl family ligases. Cbl proteins are characterized by an N-terminal tyrosine kinase binding (*TKB*) domain consisting of a four-helix bundle, an EF-hand (*EF*) and a Src homology 2 domain (*SH2*). This domain is connected to a really interesting new gene (*RING*) finger by a linker. The C-terminal region contains proline-rich stretches (*PR*), multiple serine and tyrosine residues, and a leucine zipper (*LZ*). *UBA* ubiquitin-associated

Kassenbrock and Anderson 2004). The mutation of this critical tyrosine residue to phenylalanine abolishes the E3 activity (Levkowitz et al. 1999). Recent NMR and small-angle X-ray scattering analyses revealed that the unphosphorylated N-terminal region of Cbl-b forms a compact structure by an intramolecular interaction, which masks the interaction surface of the RING domain with an E2 ubiquitin-conjugating enzyme (Kobashigawa et al. 2011). Phosphorylation of Y363, located in the helix linker region between the TKB and the RING domains, disrupts the interdomain interaction to expose the E2 binding surface of the RING domain (Kobashigawa et al. 2011). Because of the intramolecular interaction of the RING domain with TKB, Cbl-b is in a closed state that masks the binding site for the E2 protein. However, when Cbl-b is phosphorylated by receptor activation, the RING becomes exposed, thereby enhancing the affinity for the E2 protein (Kobashigawa et al. 2011). Thus, the phosphorylation of Y363 has been proposed to regulate the E3 activity of Cbl-b by two mechanisms: one is to remove the masking of the RING domain from the TKB domain and the other is to form a surface to enhance the binding affinity for E2 (Fig. 8.2).

8.4 Cbl-b in T Cell Activation

T cell activation is initiated by the recognition of the pathogenic or tumor antigens presented by antigen-presenting cells. Engagement of the T cell antigen receptor by the antigenic peptide triggers a cascade of signaling pathways including the phosphorylation of kinases and adaptor proteins and subcellular relocation of signal molecules and transcription factors, which eventually leads to activation of effector T cells. In addition to antigen recognition, full T cell activation requires additional signals provided by costimulatory receptors such as CD28 (Greenfield et al. 1998).

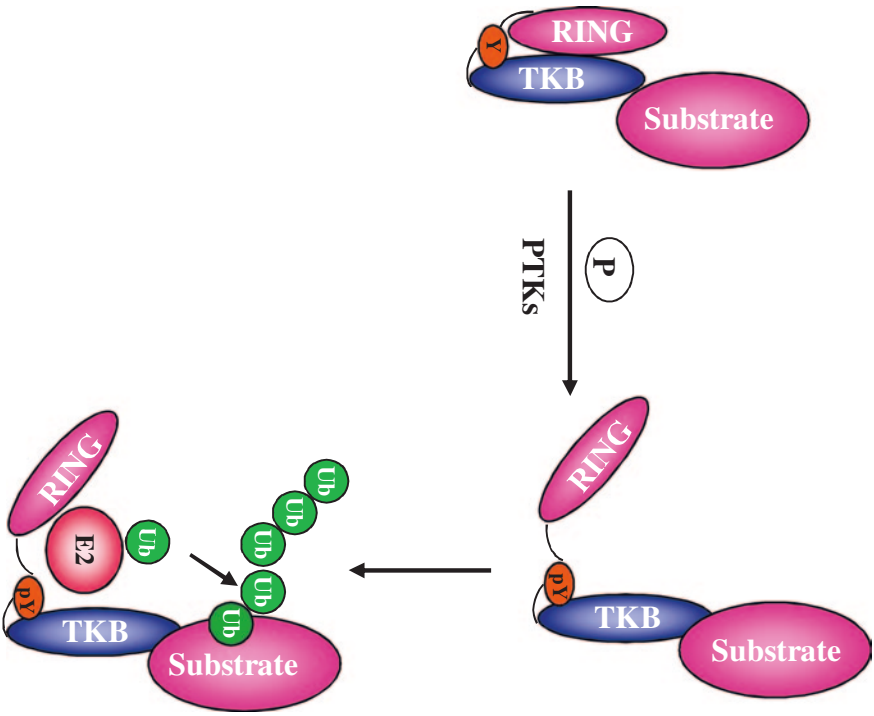

Fig. 8.2 Phosphorylation-dependent regulation of E3 ligase activity of Cbl-b. In resting conditions, Cbl-b is in a closed state and the E2 binding region of the RING domain is masked by the TKB region. On receptor stimulation, Cbl-b binds to its substrate via the TKB domain. The tyrosine kinases that are also recruited to the site phosphorylate Cbl-b at Y363. This phosphorylation-induced unmasking and conformation change enhances the affinity of the RING domain for the E2 protein, facilitating the ubiquitination of the substrate to downregulate the signals. *PTK* protein tyrosine kinase *Ub* ubiquitin

Cbl-b plays a critical negative regulatory role in both T cell receptor (TCR) and CD28 costimulatory signaling (Liu and Gu 2002; Paolino and Penninger 2010). Cbl-b$^{-/-}$ T cells produced large amounts of IL-2 and proliferated vigorously after anti-CD3 stimulation without the requirement of CD28 costimulation (Bachmaier et al. 2000; Chiang et al. 2000). Further, when CD28$^{-/-}$ mice were crossed with Cbl-b$^{-/-}$ mice, this rescued the deficiency of T-cell-dependent antibody responses caused by the CD28 deficiency, suggesting that activation of Cbl-b deficiency bypasses the requirement of CD28 costimulation (Chiang et al. 2000). Further, stimulation of the mutant T cells with anti-TCR alone induced a of Vav activation as high as that in wild-type T cells activated by anti-CD3 and anti-CD28 costimulation, suggesting that Cbl-b controls CD28 signaling through the Vav pathways (Krawczyk et al. 2000). Physiological relevance of Cbl-b-mediated regulation of T cell activation was highlighted by the observation that Cbl-b$^{-/-}$ mice are highly

susceptible to the development of spontaneous and antigen-induced autoimmune diseases (Chiang et al. 2000; Jeon et al. 2004).

8.5 Cbl-b in T Cell Tolerance

To mount a productive T cell response, TCR and costimulatory signals have to be properly integrated, which results in the genetic program that promotes transcription of effector mediators by the T cells. TCR signaling promotes phospholipase Cγ1 (PLCγ1)-mediated increase in the cellular calcium influx, which activates Ca^{2+}-dependent phosphatase calcineurin, which in turn activates the transcription factor NFAT (Rao 2009). CD28 costimulation also activates the mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways required for the activation and nuclear translocation of the transcription factors AP1 and NF-κB. These transcription factors control the transactivation of genes encoding proteins responsible for cytokine production, lineage commitment, proliferation, or migration behavior of T cells (Jain et al. 1993).

If TCR is ligated in the absence of CD28 costimulation, this results in a cell intrinsic state of functional unresponsiveness known as T cell anergy (Quill and Schwartz 1987; Schwartz 2003). Anergy induction is one of the central mechanisms to maintain immunotolerance against self-antigens (Walker and Abbas 2002; Schwartz 2003). The anergy-associated genetic program is activated if the TCR is triggered alone, partially owing to an unbalanced activation of NFAT (Macian et al. 2002). In the absence of CD28-mediated activation of AP1 and/or NF-κB, NFAT translocates to the nucleus to alternatively drive the expression of several anergy-associated genes, including cell cycle inhibitors, tyrosine phosphatases, proteinases, transcriptional regulators, diacylglycerol kinases, and importantly, Cbl-b and other E3 ligases, which actively regulate several key signaling molecules involved in T cell activation to establish the anergic state (Macian et al. 2002; Heissmeyer et al. 2004; Jeon et al. 2004; Olenchock et al. 2006; Zha et al. 2006). T cell stimulation under anergizing conditions such as stimulation with the calcium ionophore ionomycin showed a marked increase in Cbl-b expression at both the messenger RNA level and the protein level (Heissmeyer et al. 2004; Jeon et al. 2004). Cbl-b expression has also been reported in vivo on tolerance induction (Jeon et al. 2004). Genetic and biochemical studies have indicated that Cbl-b expression is dependent on the early growth transcription factors Erg2 and Erg3, which themselves are induced under anergic conditions by NFAT (Safford et al. 2005).

Anergizing stimulation such as ionomycin treatment of T cells results in a decrease in the phosphorylation of PLCγ1, with an ensuing calcium mobilization defect which is essential to mount a response (Heissmeyer et al. 2004; Jeon et al. 2004). The studies revealed that this reduced phosphorylation of PLCγ1 was not observed in Cbl-b-deficient T cells (Jeon et al. 2004). This led to the hypotheis that T cell anergy is induced and maintained via the action of Cbl-b on PLCγ1. Cbl-b upregulation in anergized T cells leads to PLCγ1 ubiquitination, resulting

in aberrant calcium signaling (Fig. 8.3). Further it was also noted that protein kinase Cθ degradation was defective in ionomycin-treated Cbl-b$^{-/-}$ T cells, suggesting that protein kinase Cθ is another substrate of Cbl-b during T cell anergic induction (Heissmeyer et al. 2004).

In addition to its role in regulation of T cell anergy induction, Cbl-b also participates in peripheral T cell tolerance by regulating resistance to regulatory T (T_{reg}) cells and transforming growth factor β (TGF-β)-induced immune suppression (Wohlfert et al. 2004, 2006; Adams et al. 2010). It has been shown that Cbl-b inhibits Smad2 phosphorylation on TGF-β in vitro stimulation (Wohlfert et al. 2006). Additionally, Cbl-b was shown to regulate Foxo1-mediated regulation of Foxp3 transcription in TGF-β-stimulated T cells (Harada et al. 2010). However, the mechanism of how Cbl-b regulates the TGF-β-induced T_{reg} cell development pathway need further detailed analysis.

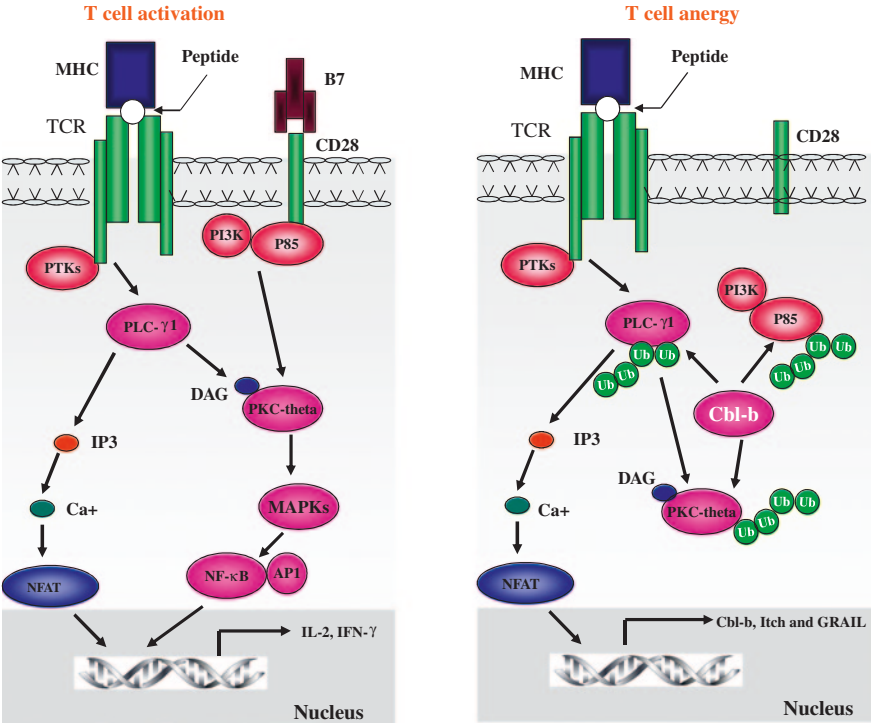

Fig. 8.3 Cbl-b-mediated regulation of T cell anergy. T cell receptor (*TCR*) signaling in combination with costimulation results in full activation of signaling pathways, resulting in Ca^{2+}-dependent activation of NFAT, activation of mitogen-activated protein kinases (*MAPKs*) and the transcription factors AP1 and NF-κB. This promotes the production of effector cytokine gene expression (*left*). Stimulation of the TCR in the absence of CD28 costimulation triggers sustained Ca^{2+}-dependent NFAT activation that leads to expression of E3 ligases Cbl-b, Itch, and Grail. Cbl-b targets phospholipase Cγ1 (*PLC-γ1*) and protein kinase Cθ (*PKC-theta*) for ubiquitination and promotes T cell anergy. *DAG* diacylglycerol, *IFN-γ* interferon-γ, *IP3* inositol 1,4,5-trisphosphate, *PI3K* phosphatidylinositol 3-kinase

8.6 Cbl-b and Antitumor Immunity

Although T cells are capable of mounting a specific response to tumor antigens, the fact that many people die of cancer every year clearly suggests the T cell response to tumors is often ineffective. This is because the cancers acquire several properties to overcome the immune surveillance mechanism of the host (Schreiber et al. 2011). Tumor antigens are often presented in the absence of costimulation, resulting in T cell anergy, and tumors secrete the inhibitory cytokine TGF-β (Massague 2008) and create an immune-suppressive tumor microenvironment, which supports their growth. The tumor-derived TGF-β not only inhibits T cell function but also promotes the development of tumor-antigen-specific induced T_{reg} cells (Colombo and Piconese 2007; Liu et al. 2007). Therefore, strategies to overcome these mechanisms could be exploited to boost the host immune response against tumors (Colombo and Piconese 2007; Qin 2009).

Because Cbl-b$^{-/-}$ T cells are resistant to T cell anergy and TGF-β-mediated suppression, the antitumor efficacy of Cbl-b has been tested in several experimental settings. In a mouse model of implanted TC-1 tumors, Cbl-b$^{-/-}$ mice spontaneously rejected tumors (Loeser et al. 2007). Similarly, Cbl-b$^{-/-}$ mice rejected a variety of cancers, including EG7 and EL-4 and other spontaneous tumors (Chiang et al. 2007). Cbl-b$^{-/-}$ CD8+ T cells were shown to produce large amounts of IL-2 and interferon-γ, resulting in enhanced expansion of immunodominant CTL clones (Chiang et al. 2007; Loeser et al. 2007). This suggests the possibility of promoting antitumor immunity by targeting Cbl-b in CD8$^+$ T cells even in the context of ineffective costimulation, impaired CD4$^+$ T cell help, or a suppressive tumor microenvironment.

8.7 Conclusion and Perspectives

Studies over the last few years have clearly established the central role of E3 ligase Cbl-b as a "gatekeeper" of T cell activation and tolerance. Cbl-b regulates T cell activation by ubiquitinating key T cell signaling molecules directly involved in the TCR and CD28 signaling pathways. When TCR is ligated in the absence of CD28 costimulation, Cbl-b along with the other E3 ligases promotes and maintains T cell anergy. Detailed studies focusing on the fundamental molecular mechanism of Cbl-b-regulated T cell response could lead to novel therapeutic approaches, wherein Cbl-b-specific activators/inhibitors could serve in the treatment of inflammatory disorders such as cancer and autoimmune diseases.

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Chapter 9
BH3-Only Proteins in Cancer and Apoptosis

Fabio Ghiotto, Claudya Tenca, Franco Fais and Silvia Bruno

Abstract In multicellular organisms, an enormous number of cells die every day, because of stress, injury, infection, or natural turnover necessary for proper tissue homeostasis. In most of these events, cell death is orchestrated by the dying cell itself, a sort of cellular suicide called "apoptosis". Malfunctioning of this death program at any stage leads to severe human diseases, including cancer and autoimmune disorders. Apoptosis is elicited by intracellular or extracellular stimuli that activate a common cell death machinery, culminating in permeabilization of the mitochondrial membrane. In this chapter we describe the state of the art of the knowledge of how cells execute "mitochondrial" or "intrinsic" apoptosis. A fundamental and crucial role as regulators of the cell's commitment to apoptosis is assumed by BH3-only proteins, a class of small molecules belonging to the Bcl-2 family. They are both sensors of death signals and vectors of information to the core apoptotic machinery, exerting their activity by hierarchical and finely tuned interactions with the other Bcl-2 family members. We discuss the groundbreaking research from several laboratories that have contributed to disclosing the complex activity of BH3-only proteins and the efforts made to translate these results into novel tools for cancer therapy.

Keywords Apoptosis • Mitochondrial dysfunction • Bcl-2 family • BH3-only proteins • BH3 mimetics in cancer therapy

9.1 Mitochondrial Dysfunction and Apoptosis

The intrinsic apoptotic pathway travels across the mitochondrion to commit the cell to programmed cell death. Mitochondria collect and integrate opposite proapoptotic and antiapoptotic signals arising from endogenous or exogenous factors. When

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death-inducing signals predominate over life-preserving ones, mitochondria eventually undergo membrane permeabilization. Specifically, the point of no return along this pathway of apoptosis is achieved when the mitochondrial outer membrane (MOM) becomes permeabilized and is the key event that irreversibly commits the cell to death.

In healthy cells the MOM is permeable to small metabolites and solutes up to approximately 5 kDa, by virtue of specific protein channels. But when its permeability increases above this threshold, soluble proteins retained within the mitochondrial intermembrane space can be released into the cytosol. Among these proteins are crucial proapoptotic factors that trigger irreversible biochemical and morphological cell death events. These include one important component of the mitochondrial respiratory chain, namely, cytochrome *c*: when translocated to the cytoplasm, it stimulates the assembly of a multiprotein complex known as the Apaf-1 apoptosome that recruits and activates caspase 9, initiating a cascade of effector caspase activation (Shi 2006).

Permeabilization of the MOM requires the close connivance of two crucial molecules, Bcl-2-associated X protein (Bax) and Bcl-2-antagonist/killer protein (Bak). These are members of the Bcl-2 family and are the proapoptotic effectors. In the inactive state, Bax loiters in the cytoplasm, as a monomer, until it is needed on the mitochondrion (Hsu et al. 1997), whereas Bak is loosely associated with the mitochondrial membrane (Wei et al. 2000). On activation, Bax translocates to mitochondria, Bak and Bax undergo conformational modification (Korsmeyer et al. 2000; Reed 2006), stably insert into the MOM, form dimers or high-order homo-oligomers (Annis et al. 2005), bind to components of the permeability transition pore complex (Marzo et al. 1998; Pastorino et al. 1999), and form large lipid/protein multimeric pores on the MOM that cause functional and structural mitochondrial collapse (Schafer et al. 2009). Thus, MOM permeabilization is not an accidental process but is a tightly regulated phenomenon largely controlled by the activity of a family of evolutionarily related proteins, the Bcl-2 family.

9.2 The Bcl-2 Family

This family of proteins was named after the first of its members, Bcl-2, discovered, more than 20 years ago, at the junction of the t(14;18) chromosome translocation that hallmarks human follicular lymphoma (Bakhshi et al. 1985; Cleary and Sklar 1985; Tsujimoto et al. 1985). It was termed Bcl-2 from "B cell lymphoma 2" (McDonnell et al. 1989). The growing number of proteins related to Bcl-2 by sequence homology and involvement in the control of apoptosis that continue to be identified has led to the definition of a Bcl-2 family (Cory and Adams 2002).

Bax and Bak represent the multidomain proapoptotic subgroup of the family, and possess three (BH1, BH2, and BH3; Fig. 9.1) of the four evolutionarily conserved α-helical Bcl-2 homology (BH) domains. A third proapoptotic multidomain protein, Bcl-2-related ovarian killer (Bok), is not yet well characterized. When

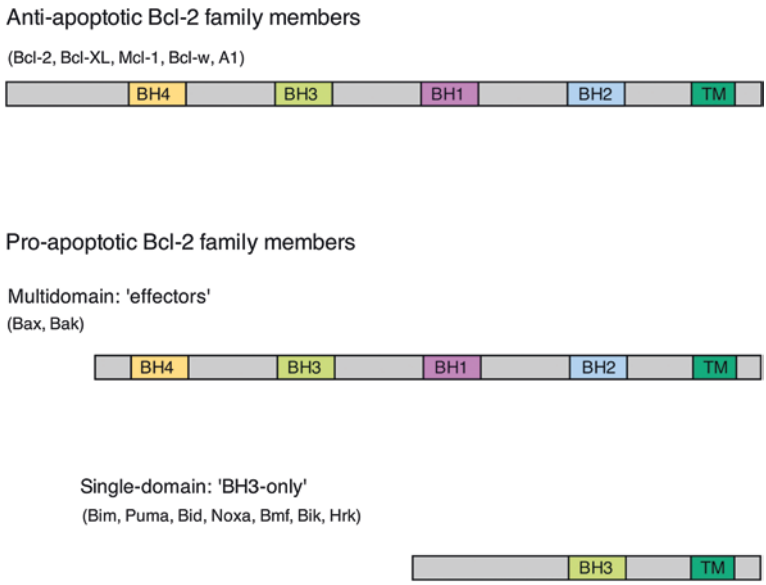

Fig. 9.1 Bcl-2 homology (*BH*) domains of the Bcl-2 family of proteins. The family is divided into antiapoptotic and proapoptotic members. The antiapoptotic members share homology in all four BH domains (BH1, BH2, BH3 ,and BH4). BH1, BH2, and BH3 comprise the Bcl-2 structural core and create the hydrophobic groove, which is the binding site for the BH3 domains of proapoptotic members. The proapoptotic members are subdivided into multidomain and single-domain proteins, i.e., "effector" and "BH3-only" proteins, respectively. The effector proteins contain homology domains BH1 to BH4, whereas the BH3-only proteins contain only one BH domain, BH3, which binds antiapoptotic proteins and, in some instances, effector proteins. Many Bcl-2 proteins also contain a transmembrane (*TM*) hydrophobic region (membrane anchor). For more details, see Hardwick and Youle (2009)

exerting their cell killing activity, these proteins form homodimers/homo-oligomers. They are essential for apoptosis, since the deletion of both genes renders cells totally resistant to cell death (Wei et al. 2001).

A second subset of the family possesses four BH domains (BH1, BH2, BH3, and BH4; Fig. 9.1) and includes five apoptosis-inhibitory proteins, i.e., the multidomain antiapoptotic proteins Bcl-2, B cell lymphoma extra large (Bcl-XL), myeloid cell leukemia sequence 1 (Mcl-1), Bcl-2-like protein 2 (Bcl-w/Bcl2L2), Bcl-2-related protein A1 (Bcl2A1), and in humans only, Bcl-B. The five antiapoptotic proteins and Bax/Bak have a similar three-dimensional structure, and the amino acid sequence signature for survival or death that distinguishes these two functional subgroups has yet to be defined. A third class of Bcl-2 proteins is composed of single-domain members that share the single BH3 motif with other Bcl-2 family members (Fig. 9.1), and were denoted as BH3-only proteins. At least eight "true" BH3-only protein have been described in mammals, including Bcl-2-interacting mediator of cell death (Bim), BH3-interacting-domain death agonist (Bid), Bcl-2-associated death promoter (Bad), Bcl-2-modifying factor (Bmf),

Noxa (the Latin word for "damage"; also known as PMAIP1), p53-upregulated modulator of apoptosis (Puma), Bik (Bcl-2-interacting killer), and Harakiri (Hrk) (Cory and Adams 2002).

The Bcl-2 family can be viewed as a tripartite switch that sets the threshold for commitment to cell death, by selective and specific interactions among members of the three Bcl-2 family subgroups. These interactions represent the key elements in the governing of MOM permeabilization and cell fate.

Despite their structural similarity, each BH3-only protein has its own pattern of interaction with antiapoptotic proteins. Some of them, such as Bim, Bid, and Puma, are promiscuous proapoptotic proteins in that they avidly bind to all antiapoptotic Bcl-2 family proteins. Others bind only to specific subsets (e.g., Bad engages Bcl-2 and Bcl-XL but not Mcl-1, and Noxa preferentially binds to Mcl-1 and not to Bcl-XL and Bcl-2) (Chen et al. 2005; Kuwana et al. 2005; Certo et al. 2006). BH3-only proteins bind the antiapoptotic proteins by inserting their BH3 domain, an amphipathic α helix, into the hydrophobic pocket made up by the folding of BH1, BH2, and BH3 domains of the antiapoptotic protein (Sattler et al. 1997). Further evidence for this interaction site came from the crystal structures of the BH3 region of Bim bound to Bcl-XL (Liu et al. 2003) and Mcl-1 (Czabotar et al. 2007), the BH3 region of Bid bound to Mcl-1 (Liu et al. 2010), and the BH3 regions of Puma, Bid, and Bmf with Bcl2A1 (Smits et al. 2008).

Like the multidomain antiapoptotic proteins, Bax and Bak bear the conventional hydrophobic BH3-binding groove. However, the roles of this binding site on Bax/Bak and its ligands are still debated. Bax and Bak seem to directly associate with antiapoptotic Bcl-2 proteins, although the binding features are much less clear, since their BH3 domain is mostly buried (Suzuki et al. 2000; Moldoveanu et al. 2006). Bak binds either Mcl-1 or Bcl-XL, but, unexpectedly, not Bcl-2 (Willis et al. 2005), whereas Bax has the capability of interacting with Bcl-2 (Zhang et al. 2004) and probably all antiapoptotic proteins (Chen et al. 2005; Willis et al. 2005, 2007).

Despite strong evidence for the functional interaction and activation of Bax and Bak by activator BH3-only proteins, their binding features have been a matter of debate for some time. However, in the last couple of years many veils have been lifted. Of the three BH3-only proteins known so far to be competent for direct interaction with Bax and Bak (and subsequent activation), i.e., Bid, Puma, and Bim (Wang et al. 1996b; Wei et al. 2000; Cartron et al. 2004; Kuwana et al. 2005; Kim et al. 2009; Mérino et al. 2009), only Bid was demonstrated to interact through its BH3 domain with the conventional hydrophobic BH3-binding groove (Lovell et al. 2008). Puma and Bim proteins may instead (or in addition) bind to a novel interaction site on Bax that involves α helices 1 and 6, distal from the conventional BH3-binding pocket, and called the "rear" site (Gavathiotis et al. 2008; Gallenne et al. 2009; Kim et al. 2009). Another study recently provided strong support for direct interaction of Bak with Bim, tBid, and surprisingly, Noxa, but through its "front site," namely, the BH3-binding pocket (Dai et al. 2011). Perhaps both sites can be used, one for promoting initial activation of Bax/Bak and the other for recruiting additional Bax (Bak) molecules (as detailed in Sect. 9.3).

Altogether, an intricate network of homodimerizations and heterodimerizations among proteins of the Bcl-2 family regulates the cell's decision to die. There is general, although not complete, agreement on the combination of interactions, but the order of these interactions and their relative importance is still a matter of debate.

A crucial role is assumed by the BH3-only protein subset of the Bcl-2 family.

9.3 How Bax/Bak Activation is Switched On or Held in Check: Models of BH3-Only Activity

BH3-only proteins are key regulators of Bax/Bak activation and orchestrate the cell's decision to undergo apoptosis. Much effort has been devoted in the last few years to understanding how they exactly accomplish these feats. Because of the profusion of Bcl-2 family members and the multiplicity of interactions between them, it has been challenging to disentangle their specific interplay during apoptosis. Protracted disputations heated up the debate on whether BH3-only members initiate apoptosis by directly binding to the cell-death effectors Bax and Bak ("direct activation" model), or whether they act indirectly, by engaging their prosurvival Bcl-2-like relatives ("indirect activation" model), until an answer came from recent studies: both mechanisms were reconciled in a novel model of transient and dynamic interactions localized at mitochondrial membranes ("dual engagement" or "embedded together" model) (Fig. 9.2).

The so-called indirect activation (or sensor-only BH3 or displacement) model postulated that antiapoptotic proteins of the Bcl-2 family normally hold in check activated or semiactivated forms of Bax and Bak by sequestering them in a reversible manner (Willis et al. 2005, 2007). According to this model, in response to apoptogenic signals, BH3-only proteins are activated, compete for the hydrophobic cleft on their respective antiapoptotic proteins, and thus allow the release of Bax and Bak, which can proceed to the completion of translocation, oligomerization, and MOM permeabilization (Adams 2003; Chen et al. 2005; Willis et al. 2005). Thus, BH3-only proteins promote apoptosis exclusively by inhibiting antiapoptotic Bcl-2 family members but are unable to directly bind and activate Bax and Bak (Willis et al. 2007).

Data from other studies, however, did not support this proposed mechanism. For instance, some investigations showed that Bax and Bak were not kept in check by antiapoptotic proteins (Kim et al. 2006) and that the promiscuous BH3-only proteins were instead capable of engaging and activating Bax and Bak (Letai et al. 2002).

An alternative model was thus proposed, the direct activation (also called "two-class" or "split BH3") model, which posited that Bax/Bak proteins are activated via interactions with a selective subset of BH3-only members, and that antiapoptotic Bcl-2 proteins act by sequestering these "activator" BH3-only proteins, rather than Bax-type proteins. Indeed, according to this model, BH3-only

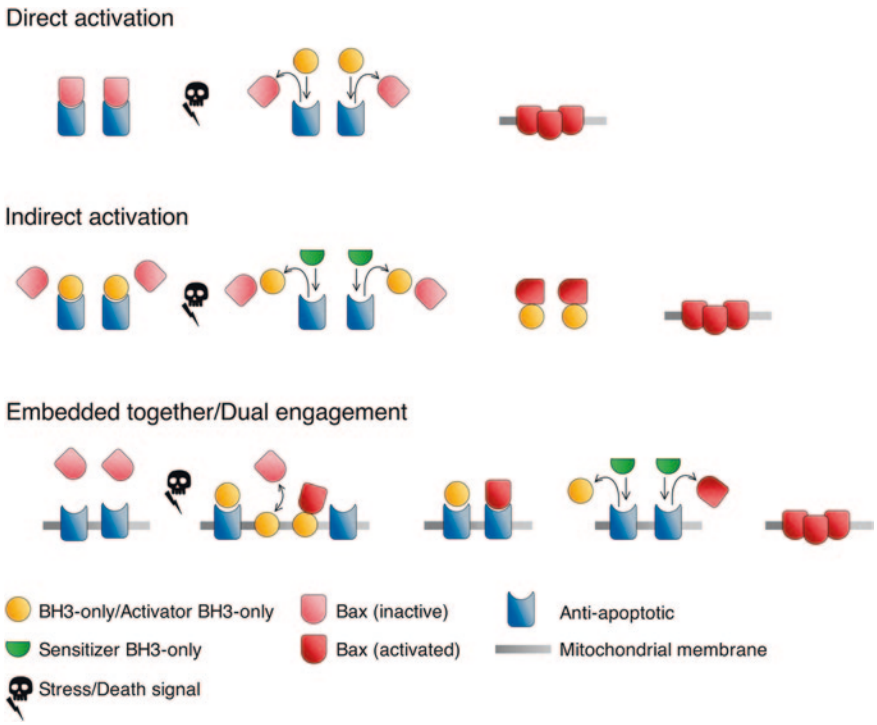

Fig. 9.2 Different models of Bax activation and oligomerization. The "direct activation" model posits that (1) healthy cells are primed for death since they bear semiactivated or activated Bax/Bak molecules held in check on the mitochondrial outer membrane by antiapoptotic Bcl-2 family members; (2) death or stress stimuli generate BH3-only proteins, which interact with their antiapoptotic relatives, according to the respective binding affinities, and induce the release of Bax by competing with the same binding sites; and (3) Bax can then proceed to complete its activation and oligomerize to form large multimeric pores on the mitochondrial outer membrane. In the "indirect activation" model the BH3-only subset is divided in two additional subgroups, according to their role with respect to Bax/Bak activation: "activator" BH3-only proteins directly bind and activate the proapoptotic effectors Bax/Bak, "sensitizer" BH3-only proteins liberate the "activators" from their block and thus permit their Bax/Bak activation function. Nonapoptotic cells reside in a "primed state" that harbors "activator" BH3-only proteins bound and sequestered by antiapoptotic Bcl-2 homologues on the mitochondrial membrane, in all possible combinations because the known "activators" are promiscuous BH3-only proteins. Bax monomers loiter in the cytosol. Apoptotic stimuli activate "sensitizer" BH3 proteins that long for antiapoptotic Bcl-2 family members, following specific combinations according to their relative binding affinities. "Sensitizers" compete for the BH3-binding hydrophobic pocket of antiapoptotic proteins, thus allowing the release of the activators. Activators can thus proceed with binding and activating Bax molecules, thus allowing their oligomerization in the mitochondrial membrane. The main novel feature introduced in the "embedded together"/"dual engagement" model is the active role of membranes for Bcl-2 family protein activation. To change their conformation, which dictates their function, Bcl-2 family proteins have to insert themselves into mitochondrial membranes. Also, important is the dynamic and transient nature of protein interaction ("kiss and run") and the fact that both classes of BH3-only proteins have a role in "activating" antiapoptotic proteins, thus causing their membrane insertion. In a nonapoptotic cell, Bax loiters in the cytosol, and antiapoptotic cells are largely anchored to mitochondrial membranes. On stress or a death signal,

BH3-only activators are generated and sequestered by antiapoptotic proteins. At low stress levels, the concentration of unbound activator BH3-only molecules is negligible and insufficient to activate Bax. Under high-stress conditions, the cellular amount of free activator BH3-only molecules increases, these molecules target mitochondrial membranes and from there they recruit cytosolic Bax, allowing its conformational change and consequent activation. However, activated Bax is immediately sequestered by antiapoptotic proteins until sensitizer BH3-only proteins are generated, and displace activated Bax and activator BH3-only molecules from their latch, allowing Bax oligomerization and pore formation

proteins are divided into two distinct subsets, "activators" (i.e., Bim, Puma, and Bid) and "sensitizers" (e.g., Bad and Noxa), each acting on a different level within a hierarchy of interactions among the various Bcl-2 proteins. In response to apoptogenic signals, the activator BH3-only proteins, which are normally sequestered by antiapoptotic proteins, become freed from this association on displacement by sensitizer BH3-only proteins that latch onto antiapoptotic Bcl-2 proteins, and compete for the BH3-binding pocket on their specific antiapoptotic targets. Thus, they prevent antiapoptotic proteins from inhibiting Bax/Bak activation by freeing activator BH3-only proteins and enable them to bind to and activate Bax and Bak (Letai et al. 2002; Chen et al. 2005; Kuwana et al. 2005; Kim et al. 2006; Walensky et al. 2006; Lovell et al. 2008). What are the activators? Bim and Bid were the first BH3-only proteins to which a direct Bax activator capability was attributed (Letai et al. 2002). Puma followed (Kim et al. 2006; Gallenne et al. 2009), although a clear picture was obtained only recently when an in vivo study using Bid, Bim, and Puma triple-knockout mice demonstrated definitively the essential role of transient and dynamic ("hit and run") activatory interactions of Bid, Bim, and Puma with Bax and Bak (Ren et al. 2010). In the absence of Bid, Bim, and Puma, Bad was unable to induce apoptosis (Ren et al. 2010). The separation line between sensitizers and activators is not well defined. In certain contexts, direct activation by BH3-only proteins other than Bim/Puma/Bid may contribute to MOM permeabilization. In the case of Noxa, for instance, some studies conclude that it is a sensitizer (Letai et al. 2002; Chipuk et al. 2005; Kim et al. 2006; Ren et al. 2010) and other results suggest that it is also a direct activator (Du et al. 2011).

In an attempt to discriminate whether the two models represented alternative paths to cell death or different stages of the same pathway, gene-targeted knock-in mice were generated in which the BH3 domain of Bim (which binds all antiapoptotic Bcl-2 proteins and Bax) was replaced with that of Bad, Noxa, or Puma (Mérino et al. 2009) so that the chimeric Bim could only bind to select prosurvival Bcl-2-like proteins: Bim^{Bad} (binding only Bcl-2, Bcl-XL, Bcl-w), Bim^{Noxa} (binding only Mcl-1, A1), and Bim^{Puma} (as a control, binding all prosurvival Bcl-2-like proteins). The results showed that, for optimal cell death, Bim must be able to bind both all antiapoptotic Bcl-2 family members (as in the indirect activation model) (Mérino et al. 2009, 2012) and Bax (as in the direct activation model) (Mérino et al. 2009), thus indicating that cell death required features of both models.

One reason which protracted the debate between defenders of the two models was the fact that Bcl-2 protein action occurs at membranes, i.e., lipid systems are more challenging to study. Most experiments conducted so far had been with either peptides/proteins in solution or with truncated proteins binding to peptides coupled to artificial surfaces. However, affinities among proteins may differ significantly when proteins are of full length and in their correct membrane topology, which allows different physiological conformations. The development of assays in reconstituted lipid-based systems (Saito et al. 2000) thus allowed the monitoring of dynamic (rather than static) interactions between Bcl-2 family recombinant proteins with lipid MOM-like liposomes or isolated mitochondria (Dlugosz et al. 2006; Leber et al. 2007; Lovell et al. 2008). The results of these studies highlighted the relevance of membranes to control conformational switches of proteins, and led to the formulation of the embedded together model (Leber et al. 2007), which reconciled and integrated aspects of the previous two models. The name came from the contention that BH3-only, Bax/Bak, and antiapoptotic proteins can exist in the embedded together configuration within MOMs. The other novel feature introduced here, not included in the other two models, was the active role of membranes in activation of Bcl-2 family proteins. To change their conformation, which dictates their function, Bcl-2 family proteins have to insert themselves into mitochondrial membranes. Several data accounted for this contention: membrane integration was necessary for Bcl-2 and Bcl-XL conformational change, which in turn was necessary for binding to membrane-embedded Bax/Bak monomers/dimers and to prevent their further oligomerization (Dlugosz et al. 2006; Leber et al. 2007); membrane integration was necessary for Bcl-XL to sequester both tBid and Bax, until the sensitizer Bad, activated by apoptotic signals, neutralized the effects of Bcl-XL by displacing tBid and Bax from Bcl-XL and promoting mitochondrial membrane permeabilization (Billen et al. 2008a; Lovell et al. 2008) (interestingly, Bcl-XL had the ability to bind and sequester both tBid and Bax equivalently in membranes, reconciling the direct and indirect activation models); also, membrane integration was necessary for tBid to be able to activate Bax, i.e., to interact with soluble Bax, and induce its insertion into the membrane and its oligomerization (Billen et al. 2008a). This latter mechanism was further confirmed for the two other activator BH3-only proteins Bim and Puma as well (Wilfling et al. 2012) according to a study that highlighted the importance of mitochondrial membrane insertion and showed that most BH3-only proteins are imported into the MOM via a C-terminal tail-anchor domain (Wilfling et al. 2012). It was proposed that the activator BH3-only molecules Bim, tBid, and Puma, from their mitochondrial tail-anchored localization, latch onto cytosolic freely diffusing Bax and trigger its well-known conformational change (Wilfling et al. 2012). This conformational change permits the mitochondrial insertion of Bax, and the process is repeated with the next Bax molecule, until enough Bax is inserted to permit oligomerization and pore formation (Wilfling et al. 2012).

Altogether, the embedded together model disclosed a scenario in which cytoplasmic antiapoptotic proteins are recruited to mitochondrial membranes where they inhibit activator BH3-only proteins and Bax/Bak to prevent MOM permeabilization unless sensitizer BH3-only proteins displace activator BH3-only proteins and Bax/Bak from

the antiapoptotic proteins to promote apoptosis (Leber et al. 2007, 2010). Thus, both proapoptotic and antiapoptotic Bcl-2 family proteins engage in reversible and dynamic interactions that are governed by membrane-dependent conformational changes and culminate in either aborted or productive membrane permeabilization.

The definitive demonstration that the direct activation model and the indirect activation model both account for efficient MOM permeabilization strategies came from a recent investigation.

A panel of chimeric activator BH3-only proteins using the backbone from the BH3-only protein Bid containing the BH3 domain from Bid itself, Bim, Bax, or Bak was generated (Llambi et al. 2011). This strategy allowed Bid to be converted from a promiscuous neutralizer of all antiapoptotic Bcl-2 proteins to a protein with differential specificity for the different antiapoptotic Bcl-2 family members, while retaining the ability to directly activate Bax and Bak. By this approach, permeabilization of the MOM was triggered under conditions where the different offensive/defensive strategies could be examined in isolation (Llambi et al. 2011). The results undoubedly demonstrated that antiapoptotic Bcl-2 family members are capable of inhibiting both activator BH3-only proteins and Bax/Bak effector proteins. Accordingly, Llambi et al. (2011) posited a model (dual engagement model) which is fully consistent with the embedded together model (Leber et al. 2007, 2010; Lovell et al. 2008). Accordingly, they proposed that Bax (cytosolic) and Bak (mitochondrial) reside in a "dormant" state in healthy cells and are not "kept in check" by antiapoptotic proteins. On apoptotic stimulation, BH3-only activators are generated and sequestered by antiapoptotic proteins. At low stress levels, the concentration of unbound activator BH3-only molecules is negligible and insufficient to activate Bax or Bak. Under high-stress conditions, the cellular amount of free activator BH3-only molecules increases, allowing Bax/Bak activation. The activated effectors are in turn sequestered by antiapoptotic protein until sensitizer BH3-only proteins displace them from their latch, allowing their oligomerization.

Altogether, the studies described above indicate that the interactions of BH3-only proteins with proapoptotic and antiapoptotic multidomain Bcl-2 family proteins can enhance, delay, or impair apoptosis depending on the relative stoichiometry and affinity of available partners in the membrane.

9.4 BH3-Only Proteins: Expression, Function, and Regulation in Cancer Cells and Cancer Therapy

There is common consensus that BH3-only proteins are essential initiators of apoptosis and propagate intrinsic and extrinsic cell death signals. The large number of BH3-only proteins, their diverse intracellular localization, the complexity of the network of specific protein interactions, and the different modes of activation ensure the flexibility needed for the cell's damage-response apparatus to regulate the transduction of different stress and damage signals to the core apoptotic machinery. Despite their structural similarity, each BH3-only protein is activated

by mechanisms that depend on the specific BH3-only protein, and include increased protein levels owing to increased transcription or decreased turnover, posttranslational modification, proteolytic cleavage, and subcellular localization. To prevent constitutive cell death, they also have to be silenced, mostly by genetic and epigenetic mechanisms.

Their antitumor activity is demonstrated by experimental mouse knockout models clearly showing that downregulation of BH3-only protein expression and activation contributes to carcinogenesis. Chemotherapies exert their cell killing functions also by upregulating and activating BH3-only proteins.

In this section we will address these aspects in detail for each of the eight best known BH3-only proteins. We will describe their proapoptotic and antitumor activity, their regulation mechanisms in tumor cells, and their involvement in cancer therapies.

Only "true" BH3-only proteins will be described, and "BH3-only-like" proteins, characterized by the possession of a single BH3 domain, will not be taken into account since their function as Bcl-2 family prodeath molecules is still obscure (Bnip3, Beclin-1, ApoL6, BRCC2, Spike, and MAP-1). Likewise, we will not address the role that some true BH3-only proteins seem to assume in other vital cellular functions not related to apoptosis regulation and cancer development.

9.4.1 Bim

In 1998 O'Connor et al. (1998) described a BH3-only protein discovered while screening a bacteriophage λ complementary DNA (cDNA) expression library using recombinant Bcl-2 as bait. The protein was termed Bim , and sequence analysis of murine cDNAs revealed the presence of three major isoforms (BimEL-196aa, BimL-140aa, and BimS-110aa) produced by alternative splicing. Shortly thereafter, a second group reported the discovery of the same gene, after screening an ovarian cDNA library using Mcl-1 as bait (Hsu et al. 1998), which they initially termed Bod (Bcl-2-related ovarian death agonist). Today, more than a dozen splice variants of Bim have been reported in mice and humans in various cell types, but their different role in cell death signaling remains largely unknown (Adachi et al. 2005).

Bim potently engages all antiapoptotic members of the Bcl-2 family with high affinity (Chen et al. 2005), and also is the most potent activator BH3-only protein that directly binds and activates Bax and Bak (Mérino et al. 2009).

Most cellular stresses, such as survival factor withdrawal, unbalance in oncoprotein expression, and endoplasmic reticulum (ER) stress, induce cellular death through Bim activation (Labi et al. 2006; Puthalakath et al. 2007). Bim appears to be particularly critical in the hematopoietic system (Villunger et al. 2003b; Labi et al. 2006). It is involved in the regulation of apoptosis and homeostasis in B and T lymphocytes and thymocytes (Labi et al. 2006). Single-allele deletion of Bim causes mice to develop Myc-induced B cell leukemia (Egle et al. 2004). Homozygous deletion of Bim is associated with mantle cell lymphoma

(Tagawa et al. 2005; Mestre-Escorihuela et al. 2007) and survival of autoreactive T and B cells (Bouillet et al. 2002; Enders et al. 2003) and lymphomas resistant to cytokine deprivation (Bouillet et al. 1999). MicroRNAs contribute to tumorigenesis by downregulating Bim gene expression (Terasawa et al. 2009). Development of Burkitt lymphoma is favored by EBV-mediated repression of Bim expression (Anderton et al. 2008) or downregulation of Bim by promoter hypermethylation (Mestre-Escorihuela et al. 2007). Tumor development and chemoresistance of cancer cells can be promoted by oncogenic protein kinases such as mutant B-Raf or N-Ras through the reduction of Bim activity (Kuroda et al. 2006; Oliveira et al. 2007; Cragg et al. 2008). Downregulation of Bim expression triggers resistance to various chemotherapeutic agents, including imatinib (Kuroda et al. 2006), paclitaxel, and bortezomib (Tan et al. 2005; Cragg et al. 2009). In fact, several antitumor chemotherapies kill neoplastic cells through Bim-activated pathways. These include DNA synthesis inhibitors (Sinicrope et al. 2008b), DNA damaging agents (Zantl et al. 2007), proteasome inhibitors (Fennell et al. 2008), epidermal growth factor receptor inhibitors (Cragg et al. 2007), kinase inhibitors (Kuroda et al. 2006; Zhang et al. 2008), histone deacetylase inhibitors (Gillespie et al. 2006), microtubule inhibitors (Tan et al. 2005; Janssen et al. 2007), glucocorticoids (Erlacher et al. 2005; Iglesias-Serret et al. 2007), and ABT-737 (Del Gaizo Moore et al. 2007).

Bim is constitutively expressed in several tissues (O'Reilly et al. 2000) and is regulated at both the transcriptional and the posttranslational level (Labi et al. 2006). Transcription can be induced by the transcription factor forkhead box O3a (Dijkers et al. 2000) and the MEK/ERK pathway (Reginato et al. 2003). Regulation of Bim activity is associated with subcellular localization and phosphorylation features. Normally, Bim is localized to the microtubules by binding cytoplasmic dynein light chain and is therefore sequestered to the microtubule-associated dynein motor complex (Puthalakath et al. 1999). In response to death stimuli, Bim becomes released from the dynein motor complex on c-Jun N-terminal kinase (JNK)-mediated phosphorylation (Puthalakath et al. 1999, 2001) and is allowed to translocate to mitochondrial and ER membranes. Downregulation of Bim expression is regulated by proteasomal degradation after ERK-mediated phosphorylation and subsequent ubiquitination (Akiyama et al. 2003). Inhibition of the latter mechanism is used by some antitumor chemotherapies, such as the kinase inhibitor imatinib, which trigger the apoptotic activity of Bim not only through transcriptional activation but also through inhibition of its proteasomal degradation (Kuroda et al. 2006).

9.4.2 Puma

Puma (also known as Bcl-2 binding component 3, bbc3) was identified in 2001 simultaneously in three independent laboratories (Han et al. 2001; Nakano and Vousden 2001; Yu et al. 2001) through profiling of genes that were expressed after p53 expression, differential messenger RNA (mRNA) expression in cells exposed to

apoptotic stress, or interaction cloning (Han et al. 2001; Nakano and Vousden 2001; Yu et al. 2001). Two laboratories in Maryland (Nakano and Vousden 2001; Yu et al. 2001) reported the discovery of this BH3-only molecule in the same journal issue and termed it Puma), and a third group in Massachusetts reported the discovery in another journal and termed this BH3-only molecule bbc3 (Han et al. 2001).

All three groups demonstrated that Puma/bbc3 is a direct target of p53 through consensus p53 binding sites within its promoter region (Han et al. 2001). Puma accounts for virtually all of the proapoptotic activity of p53, along with another BH3-only protein, Noxa, which has a minor function (see Sect. 9.4.5). Among over a dozen p53 downstream targets implicated in regulating apoptosis (Yu and Zhang 2005), only deficiency in Puma results in striking phenotypes, leaving no doubt that it is an essential mediator of p53-dependent apoptosis.

In addition to its p53-dependent apoptotic activity, Puma/bbc3 was suggested by Han et al. (2001) to be induced also by p53-independent apoptotic stimuli. Thereafter, p53-independent transcription mechanisms were confirmed in several contexts and experimental apoptosis models (Jeffers et al. 2003; Yu and Zhang 2008).

Puma interacts with high affinity with all prosurvival Bcl-2 family members (Chen et al. 2005; Kuwana et al. 2005; Certo et al. 2006). In recent years Puma was recognized to be not only a sensitizer but also a direct activator BH3-only protein since, along with Bim and Bid, it is able to directly activate Bax and Bak (Gallenne et al. 2009; Kim et al. 2009). Indeed, like Bim, Puma is a very potent BH3-only protein in many contexts, also because of its ability to promiscuously interact with all five antiapoptotic Bcl-2 family members. Nevertheless, possibly owing to the abundance of different BH3-only molecules, inactivation of Puma alone is not tumorigenic on its own, but provides transformed cells with a strong advantage. Indeed, Puma-deficient mice do not develop tumors with age (Jeffers et al. 2003), but display striking apoptotic deficiencies (Jeffers et al. 2003; Villunger et al. 2003a). Puma-knockout colon cancer cells were highly resistant to apoptosis induced by p53 overexpression or the DNA damaging agents adriamycin, 5-fluorouracil (5-FU), cisplatin, etoposide, camptothecin, and UV and γ irradiation (Yu et al. 2003; Chipuk et al. 2005; Ding et al. 2007; Wang et al. 2007). Inactivation of Puma increased lymphoma formation in Myc transgenic mice (Michalak et al. 2010) and tumorigenicity of E1A/ras-transformed mouse embryonic fibroblasts in nude mice (Hemann et al. 2004; Garrison et al. 2008) and rendered mouse embryonic fibroblasts (Villunger et al. 2003a) and p53-deficient lung cancer cell lines (Yu et al. 2006) refractory to several chemotherapeutic drugs. Loss of Puma in Bim-deficient mice exacerbated hyperplasia of lymphatic organs, and promoted spontaneous malignancies (Erlacher et al. 2006). Apoptosis normally induced by withdrawal of cytokines was blocked in myeloid cells (Jeffers et al. 2003; Ekert et al. 2006) and mast cells (Ekoff et al. 2007) from Puma-knockout mice. Likewise, Puma knockdown in vitro suppressed serum-starvation-induced apoptosis in leukemia cell lines (Ming et al. 2008).

Thus, according to the above-mentioned studies conducted on models of oncogene-driven lymphomagenesis or carcinogen-driven tumor formation, Puma is well considered a tumor suppressor. Nevertheless, recent studies on irradiation-driven

tumor formation provided surprising data: Puma promoted, instead of inhibiting, the formation of radiation-induced thymic lymphomas, and this was due to apoptosis of the hematopoietic stem cells (Labi et al. 2010; Michalak et al. 2010). Normally, thymocytes of wild-type (Puma-competent) mice undergo massive radiation-induced apoptosis, which is p53- and Puma-dependent (Jeffers et al. 2003). Absence of Puma protects cells from this radiation-induced apoptosis (Erlacher et al. 2005), and p53-deficient mice develop thymic lymphomas when repeatedly exposed to low-dose radiation (Kemp et al. 1994). Thus, one would expect that loss of Puma would accelerate radiation-induced transformation. Instead, $Puma^{-/-}$ mice clearly resisted radiation-induced lymphomagenesis (Labi et al. 2010; Michalak et al. 2010). The concept of "compensatory proliferation" was used to explain this paradox, according to a recent study describing a mechanism for apoptosis-induced compensatory proliferation of stem cells. This study showed that apoptotic cells provide proliferation signals to neighboring stem cells in a paracrine manner, thus stimulating tissue regeneration (Li et al. 2010). Accordingly, the following model was proposed to explain the "Puma paradox" of Puma-reinforced tumor formation. In wild-type mice, γ-irradiation simultaneously induces massive apoptotic cell death in the hematopoietic compartment and oncogenic mutations in the surviving population. Leukocyte depletion and surrounding apoptotic cells foster compensatory proliferation of the hematopoietic stem/progenitor cells, and those harboring mutations that outcompete nonmutated cells may then acquire secondary mutations, eventually leading to malignant transformation. In the absence of Puma, cell loss via apoptosis is strongly impaired, and no compensatory proliferation follows. These results revealed an unexpected role of Puma in stem cell homeostasis after DNA damage and cellular transformation driven by induction of apoptosis.

Puma is normally expressed at a very low level (Yu et al. 2001), but is rapidly induced in response to a wide range of stresses of virtually every tissue origin that has been examined. Growth factor or cytokine withdrawal (Han et al. 2001; Ming et al. 2008), kinase inhibition (Villunger et al. 2003a; Wang et al. 2007), ER stress (Reimertz et al. 2003; Futami et al. 2005), and oxidative stress and generation of reactive oxygen species (ROS) (Macip et al. 2003; Steckley et al. 2007; Li et al. 2008) are p53-independent Puma inducers. However, it is well known that ROS are generated during Puma-mediated apoptosis as a result of mitochondrial damage (Liu et al. 2005). It was proposed that induction of Puma by ROS may provide a feed-forward mechanism for signal amplification in the execution of apoptosis. Induction of Puma by ROS is mediated by multiple transcription factors, including p53. Most of the other p53-dependent pathways that upregulate Puma expression are triggered by DNA damage resulting from γ-irradiation (Jeffers et al. 2003; Villunger et al. 2003a; Qiu et al. 2008) and a variety of genotoxic agents, many of which are conventional chemotherapeutic drugs. Indeed, most antitumor chemotherapies kill neoplastic cells through Puma-activated pathways. These include kinase inhibitors (Dudgeon et al. 2012), DNA damaging agents (Nakano and Vousden 2001; Villunger et al. 2003a; Erlacher et al. 2005), glucocorticoids (Erlacher et al. 2005), the epidermal growth factor receptor inhibitors gefitinib and erlotinib (Sun et al. 2009), and the BH3 mimetic gossypol (Meng et al. 2008).

DNA synthesis inhibitors also kill neoplastic cells through Puma upregulation (Sinicrope et al. 2008b) In particular, increased expression of Puma is associated with better prognosis in patients receiving 5-FU-based therapy in stage II and III colon cancer, and is an independent prognostic marker for overall and disease-free survival (Sinicrope et al. 2008b). Puma also mediates TNF-α-induced apoptosis both in vitro and in vivo (Wang et al. 2009), and in Puma-deficient mice TNF-α-induced apoptosis was markedly reduced (Wang et al. 2009). Since TNF-α-induced apoptosis is mediated by interactions between TNF-α and cell-surface receptors, thus eliciting the extrinsic apoptotic pathway, the two pathways (extrinsic and intrinsic apoptotic pathways) may talk to each other not only through the actions of Bid (see Sect. 9.4.3), but also through Puma.

Puma upregulation and apoptosis in human cancer cells, elicited by chemopreventive agents such as celecoxib (Ishihara et al. 2007), green tea polyphenol (Wang et al. 2008), and the synthetic retinoid analogue 4-hydroxybenzylretinone (Anding et al. 2007), is mostly p53-independent.

In cancer cells the function of Puma appears to be compromised. The chromosomal locus 19q13.3, harboring the Puma gene, is frequently lost in human tumors (Yong et al. 1995; Mora et al. 2001). The expression of Puma itself is reduced in malignant cutaneous melanoma, and Puma expression is an independent predictor of poor prognosis in melanoma patients (Karst et al. 2005). Approximately 40 % of Burkitt lymphomas do not express detectable Puma levels (Garrison et al. 2008). Although in some tumors this is associated with DNA methylation (Garrison et al. 2008), the mechanisms of Puma downregulation in tumor cells have not been fully elucidated. Nevertheless, negative regulation of Puma transcription was proven to be mediated by transcriptional repressors such as Slug (Wu et al. 2005), alternative splice products of p73 (Melino et al. 2004) or microRNA (Choy et al. 2008). Importantly, more than half of human tumors contain p53 mutations (Vogelstein and Kinzler 2004), which may abrogate the induction of Puma by irradiation and many chemotherapeutic drugs (Yu and Zhang 2005). No mutation of the Puma gene itself has been found so far in the several tumors analyzed (Hoque et al. 2003; Yoo et al. 2007; Ahn et al. 2008).

The Puma gene encodes two BH3-domain-containing proteins (Puma α and Puma β), which show similar activities. Additional splice variants have been identified, some of which lack the BH3 domain, and whose functions are currently unclear. Puma is expressed ubiquitously in the several tissues analyzed and is targeted to mitochondria by a C-terminal hydrophobic domain (Nakano and Vousden 2001; Yu et al. 2003). Once inserted in the MOM, Puma is able to activate extramitochondrial Bax without the requirement of other Bcl-2 family members (Wilfling et al. 2012).

To date, only transcriptional induction of Puma has been reported. Among the transcription factors that activate Puma, the most relevant is p53, which is recruited to the two p53-responsive elements in the Puma promoter within a few hours of the DNA damage (Wang et al. 2007). In addition to p53, a number of other p53-independent transcription factors are implicated in Puma induction, and include the transcription factors p73 (Melino et al. 2004; Ming et al. 2008; Sun et

al. 2009), forkhead box O3a (Dudgeon et al. 2010), and nuclear factor κB (Wang et al. 2009; Dudgeon et al. 2012). C/EBP homologous protein and E2F1 are involved in Puma induction following ER stress (Li et al. 2006). The oncoproteins E2F1 and c-Myc can induce Puma through their respective binding sites in the Puma promoter (Fernandez et al. 2003; Hershko and Ginsberg 2004). Moreover, general transcription factors, including C/EBPβ and CREB (Qiao et al. 2003), c-Jun (Hayakawa et al. 2004), and Sp1 (Koutsodontis et al. 2005) have been implicated in Puma induction.

9.4.3 Bid

Bid was first cloned in 1996 on the basis of its binding to both Bcl-2 and Bax (Wang et al. 1996b), and was identified again 2 years later as a substrate of caspase 8, the apical caspase in the death-receptor apoptotic pathway (Li et al. 1998; Luo et al. 1998). It binds with high specificity all multidomain Bcl-2 family members, whether prosurvival or proapoptotic (Kuwana et al. 2005; Certo et al. 2006). Although Bid is a BH3-only protein that shares significant sequence similarities within its BH3 domains with the other BH3-only proteins, it displays some unique properties: unlike all other known BH3-only proteins, Bid needs to be proteolysed (Li et al. 1998; Luo et al. 1998) to exert potent proapoptotic activity, it exerts some functions in the nucleus as well (Liu et al. 2011), it displays a certain level of helical structure in the BH3 domain (McDonnell et al. 1999), and it functions not only for apoptosis but also in the DNA-damage response (Zinkel et al. 2005). Each of these features is detailed below.

Bid is the only BH3 protein known so far that needs to be cleaved to exert its apoptotic function. The full activity of Bid is not realized until it has been cleaved proteolytically by multiple proteases, including caspases (Li et al. 1998; Luo et al. 1998). Thus, since caspase 8 is one of the most relevant effectors of the death-receptor (or extrinsic) apoptotic pathway, the fact that Bid can be activated by caspase 8 provides a means by which the extrinsic apoptotic pathway triggers the intrinsic one, linking the death-receptor pathway to the permeabilization of the MOM (Li et al. 1998; Luo et al. 1998). After the triggering of the extrinsic apoptotic pathway by death receptors, the activated caspase 8 cleaves Bid, which normally resides in the cytoplasm, to a C-terminal fragment, tBid, allowing the exposure of a glycine residue that becomes N-myristoylated. This modification makes Bid capable of translocating to mitochondria, and inserting itself into and accumulating on mitochondrial membranes, where tBid drives the translocation and insertion of Bax into the MOM (Eskes et al. 2000), eventually leading to Bax/Bak-dependent MOM permeabilization (Wei et al. 2000; Letai et al. 2002; Billen et al. 2008b). This ordered series of events required for MOM permeabilization was demonstrated in an elegant study by Lovell et al. (2008). The same study also showed that Bcl-XL prevents membrane-bound tBid from binding Bax, whereas Bad releases tBid from Bcl-XL, restoring both tBid binding to Bax and membrane permeabilization (Lovell et al. 2008).

Recruitment of cytosolic Bax by tBid does not require other Bcl-2 family members (Wilfling et al. 2012). However, Bid does not mediate all signaling from death receptors. Indeed, Bax could be activated after treatment with TNF-α also in *Bid*$^{-/-}$ cells (Ruffolo et al. 2000). Recent experiments on *Bid*$^{-/-}$ mice have demonstrated the cell type specificity of Bid's cooperation with the extrinsic apoptotic pathway: for still unknown reasons, Bid is critical for Fas-induced apoptosis in certain cell types (so-called type 2 cells), including hepatocytes and pancreatic β cells, but is dispensable in others (so-called type 1 cells), including thymocytes and mature T cells (Kaufmann et al. 2007; Jost et al. 2009).

Under certain conditions, e.g., in cells under replicative stress, the remaining small N-terminal fragment removed from Bid may associate with the DNA damage sensor complex on chromatin and contribute to control the DNA-damage response (Liu et al. 2011). Thus, Bid is a sentinel for both DNA damage (in the nucleus) and external death signals (on mitochondria).

Most BH3-only proteins can be classified as intrinsically unstructured proteins (Hinds et al. 2007). This is not true of Bid (and Bik). Bid contains a constitutively helical BH3 region (McDonnell et al. 1999), which makes Bid's structure similar to that of multidomain Bcl-2 proteins, i.e., similar to that of proapoptotic Bax (Suzuki et al. 2000) and Bak (Moldoveanu et al. 2006), and antiapoptotic Bcl-2 proteins. Bid does not contain a C-terminal transmembrane region, and membrane insertion after cleavage by caspase 8 or other proteases is dependent on the central helices (Lutter et al. 2000; Kim et al. 2004b). High similarities between Bid and Bax were also observed in terms of migration to and insertion into the MOM, which are different from those in the other BH3-only proteins (Billen 2008b), suggesting that Bid may be conceptually more similar to Bax than it is to BH3-only proteins. However, Bid does not form oligomers and reacts differently from Bax in the presence of detergents and membranes, highlighting clear differences in the modes of action of the two proteins (Bleicken et al. 2012).

Cardiolipin, a mitochondrial lipid particularly abundant in the mitochondrial inner membrane (Lutter et al. 2000; Kim et al. 2004b), and the protein MTCH2/MIMP (Zaltsman et al. 2010) are binding factors that facilitate tBid membrane binding. Importantly, the specific binding to cardiolipin occurs through the αH6 helix and is independent of its BH3 domain (Gonzalvez et al. 2010). After tBid has bound to cardiolipin mostly at the contact sites between the MOM and the mitochondrial inner membrane, mitochondrial bioenergetics is disrupted (Gonzalvez et al. 2010). Only then does tBid recruit and activate Bax/Bak through its BH3 domain. Thus, tBid may trigger apoptosis through a double-step mechanism: first, it primes mitochondria via its αH6 helix, and then it promotes Bax/Bak activation and oligomerization through its BH3 domain.

Whether loss of Bid expression can contribute to tumorigenesis and/or drug resistance in humans has only been poorly investigated. One extensive immunohistochemical survey in a variety of different tumors revealed that Bid expression is rarely decreased in cancer cells (Chen et al. 2001; Krajewska et al. 2002) and is very rarely inactivated by gene mutation (Lee et al. 2004). Immunohistochemical analysis of Bid expression in hepatocellular carcinoma specimens indicated

weaker, albeit clearly detectable expression levels in tumor tissue when compared with matched nontumor tissue (Chen et al. 2001). Nevertheless, an important role in maintaining myeloid homeostasis and suppressing leukemogenesis was attributed to Bid on the basis of the observation that Bid-deficient mice accumulate chromosomal aberrations and develop fatal myeloproliferative disorders that resemble chronic myelomonocytic leukemia (Zinkel et al. 2003).

Bid-null mouse embryonic fibroblasts are more resistant than are wild-type fibroblasts to the DNA damaging agent adriamycin and the nucleotide analogue 5-FU (Sax et al. 2002). Several antitumor chemotherapies kill neoplastic cells through Bid-activated pathways. These include DNA synthesis inhibitors (Lee et al. 2004; Sinicrope et al. 2008a) and DNA damaging agents (Zinkel et al. 2005; Kohler et al. 2008; Pradhan et al. 2008; Song et al. 2008; Shelton et al. 2009).

As opposed to the conventional view of Bid as a proapoptotic protein, recent data demonstrated that, under certain circumstances, Bid can also exert a protective role. Bone marrow transplantation with hematopoietic cells from $Bid^{-/-}$ or $Bid^{+/+}$ mice indicated that the lack of Bid significantly reduced the reconstitution of donor hematopoietic cells, demonstrating a positive impact of Bid on hematopoietic cell repopulation under the stress condition of bone marrow transplantation (Shen et al. 2011). Bid can also act to induce cell cycle arrest and inhibition of apoptosis in the DNA damage response (Kamer et al. 2005; Zinkel et al. 2005) through phosphorylation mediated by the ATM kinase and occurring on two ATM consensus sites on Bid (Kamer et al. 2005; Liu et al. 2011). ATM-mediated phosphorylation of Bid protected hematopoietic stem cells from irradiation, regulating their quiescence and survival (Maryanovich et al. 2012). Accordingly, the ATM–Bid pathway appears to serve as a critical checkpoint for coupling hematopoietic stem cell homeostasis and DNA damage stress response to make possible long-term regenerative capacity (Maryanovich et al. 2012). A survival function of Bid in hematopoiesis was observed also in the setting of chronic replicative stress (Liu et al. 2012), as Bid protected murine hematopoietic cells during the response to long-term replicative stress induced by hydroxyurea treatment (Liu et al. 2012). The regulatory network of these protective mechanisms has to be fully disclosed.

Bid expression is transcriptionally upregulated by p53. The Bid genomic locus contains p53-binding DNA response elements (Sax et al. 2002) and its mRNA level is increased in a p53-dependent manner in vitro and in vivo (Sax et al. 2002). At the posttranslational level, Bid is activated by proteolytic cleavage. In addition to caspase 8, other proteases can cleave Bid, such as granzyme B (Barry et al. 2000; Heibein et al. 2000; Sutton et al. 2000; Cullen et al. 2007), cathepsins (Droga-Mazovec et al. 2008), and calpains (Mandic et al. 2002).

9.4.4 Bad

Bad was the first BH3-only protein to be discovered as a binder of the Bcl-2 family antiapoptotic proteins (Yang et al. 1995; Wang et al. 1996a; Datta et al. 1997).

The first structural studies on complexes between peptides containing BH3-helix-containing peptides and different antiapoptotic proteins demonstrated that Bad interacted with high affinity with Bcl-XL (Petros et al. 2000). Further in vitro binding assays revealed Bad to be a heterobinder to Bcl-2, Bcl-XL, and Bcl-w but not Mcl-1 (Letai et al. 2002; Chen et al. 2005; Kuwana et al. 2005). It is considered a sensitizer BH3-only protein, since no direct interactions with Bax/Bak have been evinced.

A plethora of in vitro studies demonstrated the proapoptotic functions of Bad in different cell types and contexts. In vivo investigations revealed its antitumor activity. Loss of Bad rendered cells independent of growth factors for survival (Ranger et al. 2003) and with age Bad-knockout mice developed diffuse large B cell lymphoma of germinal center origin (Ranger et al. 2003). Multiple myeloma cells displayed gene-methylation-induced decrease of Bad expression (Pompeia et al. 2004). Bad loss is tumorigenic in mice and in humans. Bad is negatively regulated by phosphatidylinositol 3-kinase/Akt signaling (Datta et al. 1997), a survival pathway frequently upregulated in many human tumor types. However, this BH3-only protein does not seem to have a very crucial role in cell death, possibly because other more potent BH3-only proteins exert their proapoptotic role, such as Bim, whose function partly overlaps with that of Bad. As an example, Bad-deficient cells remain sensitive to cytokine deprivation and other cytotoxic stimuli (Kelly et al. 2010). Nevertheless, several antitumor chemotherapies kill neoplastic cells through Bad-activated pathways. These include DNA synthesis inhibitors (Sinicrope et al. 2008a), epidermal growth factor receptor inhibitors (Gilmore et al. 2002), and kinase inhibitors (Kuroda et al. 2006; Zhang et al. 2008), which mostly activate Bad by dephosphorylation (Kuroda et al. 2006).

The activity of Bad is mainly regulated at a posttranslational level by phosphorylation and dephosphorylation mechanisms, which modulate its subcellular localization and activation state (Ayllon et al. 2002; Polzien et al. 2009, 2011). Various phosphorylation sites on Bad influence its localization and its interaction with other proteins (Polzien et al. 2009, 2011). In response to apoptogenic stimuli (particularly cytokines and growth factor deprivation), endogenous Bad is dephosphorylated (Zha et al. 1996) and localizes to the MOM, where it binds to antiapoptotic Bcl-2 family proteins via its BH3 domain (Chen et al. 2005; Kuwana et al. 2005; Certo et al. 2006). The phosphatases involved in keeping Bad unphosphorylated include calcineurin (Wang et al. 1999), protein phosphatase 1 (Ayllon et al. 2000), and protein phosphatase 2A (Chiang et al. 2001). Conversely, growth factor and survival signals inactivate Bad by phosphorylation of the protein at specific serine residues (Ser-112, Ser-155, and Ser-136 in mice; Ser-75, Ser-99, and Ser-118 in humans) by protein kinases that include Akt (Datta et al. 1997), Raf-1 (Wang et al. 1996a), and protein kinase A (Harada et al. 1999). Phosphorylation of mouse Bad at Ser-112 and Ser-136 (Ser-75 and Ser-99 in human Bad) results in association of Bad with 14-3-3 scaffold proteins (Zha et al. 1996) that promote its relocation away from mitochondria to the cytoplasm and the plasma membrane (Datta et al. 2000; Ayllon et al. 2002). Phosphorylation of mouse Bad at Ser-155 (Ser-118 in human BAD) within its BH3-domain disrupts the association with Bcl-2 or Bcl-XL, promoting cell survival

(Hekman et al. 2006) and, in the presence of membranes, mediates pore formation (Polzien et al. 2011). The phosphorylation status of Bad at these serine residues reflects a checkpoint for cell death or survival.

In addition to the best known role of Bad in apoptosis, recent studies point to new roles for this protein in other physiological pathways, such as glucose metabolism (Danial et al. 2003, 2008) and central glucose sensing (Osundiji et al. 2011). We only mention that Bad was proven to regulate glucose-driven mitochondrial respiration (Danial et al. 2003) and insulin secretion by pancreatic β cells (Danial et al. 2008), and hormonal/feeding responses to hypoglycemia (Osundiji et al. 2011). Thus, it appears that Bad helps coordinate the apoptotic machinery and mitochondrial fuel metabolism.

9.4.5 Noxa

Noxa is the first BH3-only protein reported to be a p53 target. It was cloned using a differential display technique and called Noxa, the Latin word for "damage" (Oda et al. 2000). Together with Puma, Noxa accounts for all of the proapoptotic activity induced by p53 in lymphocytes with damaged DNA, as determined by studies on mice lacking both Puma and Noxa (Michalak et al. 2008).

Noxa interacts strongly only with Mcl-1 (Chen et al. 2005; Willis et al. 2005; Certo et al. 2006), and its apoptogenic activity appears to be due to Mcl-1 inhibition by targeting Mcl-1 for proteasomal degradation (Willis et al. 2005; Czabotar et al. 2007). Whether Noxa interacts selectively with the prosurvival protein BFL-1/A1 as well is still debated.

Although Noxa is considered to be only a sensitizer BH3-only protein (Letai et al. 2002; Kuwana et al. 2005; Kim et al. 2006; Ren et al. 2010), recent studies raised the possibility that Noxa acts also as a direct activator (Du et al. 2011), by binding, like Bim and tBid, to Bak through the "front site" of Bak (Dai et al. 2011).

Noxa is not a very strong apoptogenic BH3-only protein by itself. Loss of Noxa in lymphocytes and fibroblasts confers only a modest apoptosis resistance to DNA damage induced by etoposide or radiation (Shibue et al. 2003; Villunger et al. 2003a). *Noxa*$^{-/-}$ mice do not exhibit developmental abnormalities (Shibue et al. 2003; Villunger et al. 2003a). Noxa can efficiently induce cell death only if coexpressed with Bad (Chen et al. 2005) and loss of Noxa was able only to enhance the development of thymic lymphomas otherwise induced by Puma ablation (Michalak et al. 2010). A possible explanation for the apparent weakness of its apoptogenic function may be due to the fact that Noxa displays a very limited spectrum of binding to Bcl-2-like prosurvival molecules (Mcl-1). Nevertheless, despite showing weak proapoptotic potential on its own, Noxa is crucial in fine-tuning cell death decisions, because it antagonizes Mcl-1, which is almost insensitive to the action of several other BH3-only proteins, such as Bad, Bik, and Hrk.

Expression of Noxa in cancer cells becomes upregulated in response to antitumor chemotherapies such as DNA synthesis inhibitors (Sinicrope et al. 2008b),

DNA damaging agents (Villunger et al. 2003a), and fenretinide (Bruno et al. 2012), and dramatically after treatment with the proteasome inhibitor bortezomib (Velcade) (Fernandez et al. 2005; Qin et al. 2005; Fennell et al. 2008). Instead, for unknown reasons, glucocorticoid treatment of acute lymphoblastic leukemia downregulated Noxa mRNA expression and induced the proteasomal degradation of Noxa protein (Ploner et al. 2008). This phenomenon interfered with glucocorticoid-induced apoptosis (Ploner et al. 2009) and raises an issue of clinical relevance.

Like Bim, tBid, and Puma, Noxa also appears to require insertion into the MOM to exert its proapoptotic activity (Wilfling et al. 2012). Noxa mutants lacking the C-terminus did not associate with mitochondria (Wilfling et al. 2012) and the killing activity of Noxa was functional only when it was targeted to mitochondria via its C-terminal domain (Seo et al. 2003).

BH3-only molecules rarely display gene mutations. The Noxa gene was studied in several tumors and was almost never found mutated (Lee et al. 2003), except for diffuse large B cell lymphomas which displayed mutated and silenced Noxa (Mestre-Escorihuela et al. 2007).

As mentioned, the Noxa gene is a direct transcriptional target of the tumor suppressor p53 (Vousden and Lane 2007). In addition to p53, other transcription factors regulate Noxa activation. E2F1 directly upregulates the expression of Noxa, in addition to other BH3-only molecules (Puma, Bim, and Hrk/Dp5) (Hershko and Ginsberg 2004). In hypoxic condition Noxa is transcriptionally activated by hypoxia-inducible factor 1α via hypoxia-responsive elements on the Noxa promoter (Kim et al. 2004a). Adenovirus 5 E1A activates transcription of Noxa in head and neck cancer (Flinterman et al. 2005)

Noxa can be epigenetically silenced by promoter methylation and histone acetylation in different cellular contexts (Yamashita et al. 2008). Noxa protein stability is controlled by the proteasome (Fernandez et al. 2005; Qin et al. 2005; Fennell et al. 2008).

The BH3 mimetic ABT-737 holds great promise for the treatment of several tumor diseases (Del Gaizo Moore et al. 2007; Tse et al. 2008). However, its cytotoxic activity is impaired when Mcl-1 is overexpressed (van Delft et al. 2006) because ABT-737 binds to Bcl-2, Bcl-XL, and Bcl-w but not to Mcl-1 (Oltersdorf et al. 2005). Thus, high expression of Mcl-1 protects tumor cells against ABT-737, whereas approaches that downregulate Mcl-1 sensitize tumor cells to ABT-737 (see Sect. 9.5). Recent studies aimed at identifying co-treatments with ABT-737 proposed that ABT-737 should be administered with a cytotoxic agent that upregulates Noxa activity, which in turn neutralizes Mcl-1 (Konopleva et al. 2006; van Delft et al. 2006; Kang et al. 2008; Bruno et al. 2012).

9.4.6 Bik

Bik (also known as Nbk) was identified and its BH3 domain functionally was characterized in 1995 (Boyd et al. 1995). Although another BH3-only protein, namely, Bad (Yang et al. 1995), was identified slightly earlier as a Bcl-2 family

member interacting with viral and cellular antiapoptotic proteins, Bik was the first protein in which the cell death activity was linked to the conserved BH3 domain (Boyd et al. 1995; Chittenden et al. 1995) and thus is considered as the founding member of the BH3-only family proteins. Bik was shown to share two domains, the BH3 domain and the transmembrane domain, with other BCL-2 family proteins, and specific mutations within the BH3 domain abrogated the ability of Bik to induce cell death and to complex with antiapoptotic proteins (Boyd et al. 1995; Chittenden et al. 1995; Elangovan and Chinnadurai 1997; Mathai et al. 2002). The BH3 domain was necessary, but not sufficient, since an efficient proapoptotic activity of Bik required a C-terminal region (amino acids120–134) in addition to the BH3 domain (Elangovan and Chinnadurai 1997). Also, Bik carries a unique ER tail-anchor signal, characterized by an extended transmembrane domain of higher hydrophobicity at the C-terminus and the lack of flanking charged amino acid residues (Germain et al. 2002; Wilfling et al. 2012). Predictions based on the analysis of mean hydrophobicity and mean net charge suggested that Bik may have a well-defined structure, whereas most other BH3-only members (except Bid) appear to be unstructured (Hinds et al. 2007).

Bik belongs to the subclass of sensitizers, and binding with Bax or Bak has never been documented. Instead, Bik was shown to bind to antiapoptotic proteins, namely, to Bcl-XL and Bcl-w with high affinity, and to Mcl-1 and Bcl-2 with very low affinity (Chen et al. 2005; Kuwana et al. 2005; Certo et al. 2006).

As all companion BH3-only proteins, Bik is induced by various apoptotic signals, and is associated with cell death during oncogene activation and cancer progression. However, by itself, Bik seems to have a more limited role, and loss of Bik may not be greatly tumorigenic. Indeed, Bik was shown to be redundant for cell death induced by a broad range of apoptotic stimuli (Coultas et al. 2004) and apoptosis induced by these stimuli is not impaired in $Bik^{-/-}$ lymphocytes (Coultas et al. 2004). Moreover, whereas loss of the BH3-only protein Bim rescued mice lacking the prosurvival protein Bcl-2 from lymphopenia, loss of Bik did not (Coultas et al. 2004). No abnormalities appeared in $Bik^{-/-}$ mice (Coultas et al. 2004), but male mice lacking both Bim and Bik were infertile owing to impaired apoptosis of immature testicular progenitor cells (Coultas et al. 2005). These data suggest that any function of Bik in programmed cell death may overlap that of Bim and possibly other BH3-only proteins.

Bik, is strongly induced in cancer cells treated with chemotherapeutic agents, including DNA damaging agents (Real et al. 2006) and proteasome inhibitors (Fennell et al. 2008). However, although these antitumor chemotherapies appear to kill neoplastic cells through Bik-activated pathways (Real et al. 2006), the relevance of this BH3-only protein in response to cancer therapy has to be confirmed. Further functional investigations will be needed also to evaluate the relevance of Bik in cancer pathogenesis. Loss of Bik is documented in a range of different human tumor entities. As an example, malignant kidney epithelium displays decreased expression of Bik compared with the adjacent normal tissues (Sturm et al. 2006). In multiple myeloma cells, Bik is methylated (Pompeia et al. 2004). However, loss of Bik does not seem to be greatly tumorigenic by itself.

Among all BH3-only molecules, Bik displays a unique feature (in part shared by Bim) whereby it regulates mitochondrial dysfunction through the ER (Germain et al. 2002). Bik resides on cellular endomembranes, particularly around the nuclear envelope and ER by a tail-anchor sequence that mediates selective integration into the ER membrane (Germain et al. 2002; Mathai et al. 2002). Once induced, it translocates to mitochondrial membranes and/or, by direct binding to mitochondria from its location at the ER, it triggers a mitochondrial cytochrome *c* releasing activity that is independent of Bax activation (Germain et al. 2002). Bik has also been shown to induce ER-resident Bax/Bak to release Ca^{2+}, which induces the release of cytochrome *c* (Mathai et al. 2005).

Bik activity is regulated through transcriptional and posttranscriptional mechanisms. Transcriptional upregulation can be exerted by p53 (Mathai et al. 2002) or p53-independent transcription factors (Real et al. 2006; Hershko and Ginsberg 2004; Paquet et al. 2004), such as E2F1 (Real et al. 2006). Phosphorylation at residues 33 (threonine) and 35 (serine) by enzymes such as casein kinase II related enzymes, enhance Bik's apoptotic function (Verma et al. 2001), whereas mutation of these phosphorylation sites reduces its apoptotic activity (Verma et al. 2001).

9.4.7 Hrk

Hrk (also known as Dp5) was originally identified in 1997 in two different laboratories independently: on a screen for genes induced in rat sympathetic neurons and a pheochromocytoma cell line undergoing apoptosis in response to nerve growth factor (NGF) withdrawal (termed Dp5) (Imaizumi et al. 1997) and a screen of a HeLa cDNA library using Bcl-2 as bait (termed Harakiri, or Hrk) (Inohara et al. 1997).

This novel protein belongs to the sensitizer class of BH3-only proteins since it does not interact with the death-promoting proteins Bax and Bak (Inohara et al. 1997). Among antiapoptotic proteins, Hrk binds only Bcl-XL with high affinity (Certo et al. 2006) and Bcl-2 with lower affinity (Inohara et al. 1997).

Along with Bim, Hrk is the BH3-only protein mostly involved in cell death of the nervous system, because it is controlled by NGF and kills neurons of the sympathetic system when overexpressed (Imaizumi et al. 1997, 2004; Putcha et al. 2001). In a murine model, the Hrk gene expression was found to be restricted to cells and tissues of the central and peripheral nervous systems (Coultas et al. 2007), and neurons from mice lacking Hrk were less sensitive to apoptosis induced by NGF withdrawal (Coultas et al. 2007). The role of Hrk in hematopoietic tissues is more debated. Although Hrk was first found to be expressed in lymphoid tissues, particularly in bone marrow and spleen (Inohara et al. 1997), further studies indicated that Hrk is not expressed in viable hematopoietic progenitors, but it is specifically induced after growth factor deprivation (Sanz et al. 2000, 2001, 2002). Other studies reported no evidence for Hrk expression or induction in growth-factor-dependent hematopoietic cell lines following growth factor

withdrawal (Coultas et al. 2007). Also, hematopoietic progenitors in mice lacking Hrk died normally in response to cytokine deprivation (Coultas et al. 2007). It is now mostly accepted that murine Hrk is only expressed in the nervous system, whereas human Hrk has a role also in hematopoietic tissue.

Hrk is largely unstructured with residual limited α-helical conformation (Sborgi et al. 2010). However, it bears a C-terminal sequence (approximately 30 amino acids) which adopts an α-helical conformation in model membranes (Bernabeu et al. 2007) and is possibly responsible for targeting Hrk to mitochondrial and ER membranes. A recent structural study confirmed the presence of a transmembrane domain, and its three-dimensional structure suggests that membrane binding is an important determinant of Hrk's function (Barrera-Vilarmau et al. 2011). To date, "formal" transmembrane domains have been attributed to the BH3-only proteins Bik and Hrk only (Hardwick and Youle 2009).

During ceramide-induced apoptosis of oligodendrocytes, upregulated Hrk was able to directly interact with the mitochondrial pore-forming protein p32, a protein known for its role in causing mitochondrial dysfunction and cell death (Sunayama et al. 2004; Itahana and Zhang 2008). Thus, Hrk is capable of causing mitochondrial dysfunction also by directly acting with mitochondrial proteins, independent of Bax and Bak.

Hrk is downregulated by transcriptional repression due to promoter hypermethylation in several tumors, including colorectal and gastric cancer cell lines (Obata et al. 2003), prostate cancer (Higuchi et al. 2008), astrocytic tumors (Nakamura et al. 2006), and glioblastomas (Nakamura et al. 2005). Silencing of Hrk occurs more frequently in tumors with wild-type p53 (Nakamura et al. 2005), suggesting that loss of Hrk expression may relieve the pressure to lose this key tumor suppressor. However, Hrk may not be a fundamental tumor suppressor, as mice deficient in Hrk alone did not develop cancer (Coultas et al. 2007).

The levels of Hrk are upregulated by treatment with genotoxic chemotherapeutic drugs, such as etoposide (Sanz et al. 2000), doxorubicin, cisplatin, and gemcitabine (Engelmann et al. 2010).

Transcriptional upregulation of Hrk is strongly induced by E2F1 (Hershko and Ginsberg 2004; Hao et al. 2012). Activation of Hrk function mediated by JNK/c-Jun was documented in apoptosis of pancreatic β cells after different stimuli (Gurzov et al. 2009), in apoptosis of cerebellar granule neurons induced by potassium deprivation (Ma et al. 2007), and in apoptosis of oligodendrocytes on ceramide treatment (Chen et al. 2006; Rizvi et al. 2011).

Generally, the posttranscriptional downregulation of mRNA levels of the BH3-only members is the least studied of the regulatory mechanisms that control their activity. In the case of Hrk, a silencer motif in the 3′ untranslated region of the gene was found (Inohara et al. 1997) that is targeted by the transcriptional repressor DREAM after activation by prosurvival signals (Sanz et al. 2001; Hao et al. 2012), thus destabilizing Hrk mRNA.

Although Hrk might be potentially valuable for novel cancer therapy approaches, upregulation and activation of Hrk in auditory cells was shown to induce sensorineural hearing loss (Kalinec et al. 2005). Therefore, one should be

cautious when designing clinical trials with Hrk-inducing agents to foresee preventive strategies for iatrogenically induced ototoxicity (Kalinec et al. 2005).

9.4.8 Bmf

Bmf is a more recent addition to the BH3-only protein family and was originally discovered in a screen of a mouse embryonic cDNA library using Mcl-1 as bait (Puthalakath et al. 2001). Thereafter, Bmf was observed to avidly bind also Bcl-2, Bcl-XL, and Bcl-w (Chen et al. 2005).

It is the closest relative of Bim (Pinon et al. 2008). However, in contrast to Bim, little is known about the biology of Bmf. It seems that Bmf plays a more restricted role and supports Bim in some of its cell death processes (Hubner et al. 2010).

Nevertheless, Bmf is critical for apoptosis in several contexts. $Bmf^{-/-}$ lymphocytes are resistant to apoptosis induced by glucocorticoids or histone deacetylase inhibitors (Labi et al. 2008). Knockout of Bmf increases tumor load in the Eμ-myc transgenic mouse model of B cell lymphoma (Frenzel et al. 2010). Susceptibility to apoptosis of mutant B-Raf melanoma cells upon B-Raf targeting is dependent on Bmf (Shao and Aplin 2010). Bmf was even proposed as a tumor suppressor because of its chromosomal location, 15q14. Indeed, a high frequency of loss of heterozygosity at 15q14-15 was reported in several human tumors, including advanced breast, lung, and colon carcinomas (Schmutte et al. 1999). Nevertheless, no direct demonstration of the impact of Bmf as a tumor suppressor is available since the data from in vivo models are still poor.

Bmf is induced on cytokine withdrawal (Villunger et al. 2003b), in response to UV irradiation, or on loss of adhesion (anoikis condition) (Schmelzle et al. 2007), whereas Bmf knockdown prevented anoikis and promoted anchorage-independent growth (Schmelzle et al. 2007). Bmf is necessary for the effectiveness of several antitumor chemotherapies, including histone deacetylase inhibitors (Zhang et al. 2006) and kinase inhibitors (VanBrocklin et al. 2009).

Bmf is regulated in a manner similar to that for Bim. Both proteins share a conserved dynein light chain binding motif near their N-termini, and activation of the proteins is associated with subcellular delocalization. Bmf is normally bound to the cytoskeleton through dynein light chain 2, a component of the myosin V motor complex placed on the actin filament (Puthalakath et al. 2001; Day et al. 2004) and becomes released from it in response to death stimuli on JNK-mediated phosphorylation (Puthalakath et al. 1999, 2001; VanBrocklin et al. 2009). Whether control of Bmf activity is exerted at the posttranslation level as well has been less studied compared with other BH3-only proteins. However, it was shown that phosphorylation of Ser-74 by JNK (Lei and Davis 2003) and by ERK2 (Shao and Aplin 2012) and phosphorylation of Ser-77 by ERK2 (Shao and Aplin 2012) downregulate the proapoptotic activity of Bmf (Hubner et al. 2010; Shao and Aplin 2012).

9.5 BH3-Only Proteins in the Clinic: Anticancer Strategies

It is now widely recognized that ablation or inhibition of BH3-only proteins leads to apoptosis deficiency underlying increased risk of cancer development and therapeutic resistance, thus supporting a rationale for developing anticancer agents that imitate the BH3 domain of BH3-only proteins. Small molecules capable of mimicking this domain would make it possible to directly target antiapoptotic Bcl-2 family proteins, with neutralization of their functional activity. This strategy would attack upstream molecular steps leading to MOM permeabilization.

Interestingly, these BH3 mimetics may have differential cytotoxicity with regard to cancer cells with respect to normal healthy cells. Recent studies indicate higher "addiction," for survival, to antiapoptotic Bcl-2 family proteins of cancer cells than healthy cells. These studies used a tool called "BH3 profiling," which exploits the selective interaction between BH3 domains and antiapoptotic Bcl-2 family proteins (Certo et al. 2006; Del Gaizo Moore et al. 2007).

Oncogene activation, cell cycle checkpoint violation, and genomic instability are insults that normally provoke death signals. However, cancer cells violate this rule by developing apoptosis-blocking mechanisms, one of which is the increase of expression of antiapoptotic proteins that sequester large amounts of activator proapoptotic BH3-only proteins (Del Gaizo Moore et al. 2007; Del Gaizo Moore and Letai 2013). For example, cancer cells are highly sensitive to antagonists of the antiapoptotic protein function because of the consequent massive release of prodeath proteins. They are in a way "primed for death." Instead, survival of nonmalignant cells does not normally need to depend on antiapoptotic proteins. Inactivation of antiapoptotic protein function by BH3 mimetics, therefore, is not expected to exert dramatic effects on healthy cells. Although this hypothesis has not been fully demonstrated, a differential sensitivity of cancer and normal cells to BH3 mimetics has been documented in several studies.

A number of BH3 mimetic drugs have been designed in the last decade. They are either peptidic BH3 domains of BH3-only proteins or nonpeptidic synthetic small molecules with great affinity for antiapoptotic Bcl-2 family members. They were identified from random screening of natural products or designed by rational structure-based approaches. Preclinical studies have proven promising cytotoxic activity as single agents or in combination with other antineoplastic agents, and a few synthetic small molecules are now being assessed in phase I and phase II clinical trials directed against solid tumors and hematologic malignancies (Table 9.1).

The first peptidic BH3 mimetics proved to be unable to permeate into cells and to undergo rapid proteolytic degradation. Among different attempts made to stabilize the α helix, an interesting approach was developed by Korsmeyer and coworkers. The peptidic domains were "hydrocarbon-stapled" into an α-helical conformation called stabilized α helices of Bcl-2 domains (SAHB) that rendered the peptides cell-permeable and protease-resistant through the increased helicity in solution (Walensky et al. 2004, 2006; Danial et al. 2008). The SAHB peptides of Bim and Bid have shown cytotoxic activity against tumor cell lines and delayed

Tab. 9.1 Single-center and multicenter phase I and phase II clinical trials of BH3 mimetics as a single agent or in combination with other chemotherapies administered to cancer patients

Drug	Drug combination	ClinicalTrials.gov identifier	Condition	Phase	Status
(−)-Gossypol (AT-101)	SA	NCT00275431	Relapsed or refractory B cell malignancies (diverse lymphomas/chronic lymphocytic leukemia)	II	Completed
	SA	NCT00848016	Adrenocortical carcinoma (recurrent, metastatic, or primary)	II	Recruiting
	+ chemotherapy	NCT00561197	Locally advanced esophageal or gastroesophageal junction cancer	I/II	Completed
	+ temozolomide	NCT00390403	Glioblastoma multiforme	I	Completed
	+ temozolomide	NCT00773955	Extensive-stage small cell lung cancer	II	Completed
	+ docetaxel and prednisone	NCT00286793	Hormone-refractory prostate cancer	I/II	Completed
	+ androgen ablation	NCT00666666	Metastatic prostate cancer	II	Ongoing, not recruiting
	+ decetaxel	NCT01285635	Head and neck squamous cell carcinomas	II	Recruiting
Obatoclax mesylate (GX15-070MS)	SA	NCT00438178	Diverse hematologic malignancies	I	Completed
	SA	NCT00359892	Hodgkin lymphoma	II	Completed
	SA	NCT00684918	Acute myeloid leukemia	II	Completed
	SA	NCT00413114	Myelodysplastic syndromes	II	Completed
	SA	NCT00360035	Myelofibrosis	II	Completed
	+ rituximab	NCT00427856	Follicular lymphoma	II	Completed
	+ bortezomib	NCT00407303	Mantle cell lymphoma	I/II	Completed
	+ docetaxel	NCT00405951	Non-small-cell lung cancer	I/II	Completed
	+ fludarabine and rituximab	NCT00600964	B cell chronic lymphocytic leukemia	I	Completed
	+ carboplatin/etoposide	NCT00682981	Small cell lung cancer (extensive stage)	I/II	Completed

(continued)

Tab. 9.1 (continued)

Drug	Drug combination	ClinicalTrials.gov identifier	Condition	Phase	Status
ABT-263	SA	NCT00481091	Relapsed refractory chronic lymphocytic leukemia	II	Ongoing, not recruiting
	SA	NCT00408811	Leukemia, lymphoma, small intestine cancer	I/II	Recruiting
	SA	NCT00445198	Small cell lung cancer	I/II	Recruiting
	+ ketoconazole	NCT01021358	Lymphoid and solid tumors	I	Completed
	+ rifambin	NCT01121133	Lymphoid and solid tumors	I	Completed
	+ rituximab	NCT00788684	Lymphoid tumors	I	Ongoing, not recruiting
	+ rituximab (dose-intensive)	NCT01087151	Chronic lymphocytic leukemia	II	Recruiting
	+ gemcitabine	NCT00887757	Solid tumors	I	Recruiting
	+ paclitaxel	NCT00891605	Solid tumors	I	Recruiting
	+ etoposide/cisplatin	NCT00878449	Solid tumors	I	Completed
	+ erlotinib + irinotecan	NCT01009073	Various cancers	I	Ongoing, not recruiting
	+ fludarabine/ cyclophosphamide/rituximab or + bendamustine/rituximab	NCT00868413	Chronic lymphocytic leukemia	I	Recruiting
	+ bendamustine/rituximab	NCT01423539	B cell lymphoma	II	Open, not yet recruiting

SA single agent

growth of xenotransplanted human leukemia (Walensky et al. 2004). However, rather than inhibition of antiapoptotic molecules, their cytotoxic activity was mainly ascribed to direct Bax activation (Walensky et al. 2006; Gavathiotis et al. 2008; Pitter et al. 2008). Thus, they were not used for clinical studies.

Two other peptidic BH3 mimetics have been developed. One, named 072RB, was derived from the Bim BH3 domain, and specific residues were replaced with natural and nonnatural amino acids to increase its affinity for Bcl-XL, its serum stability, and its cell membrane permeability (Ponassi et al. 2008). It induces dose-dependent apoptosis of leukemic cells in vitro (Ponassi et al. 2008, Ghiotto et al. 2009) and in vivo (Ponassi et al. 2008). Still, the biochemical pathways activated by this peptide have to be fully demonstrated. Another peptidomimetic, a synthetic heterocyclic compound named S1, inhibits Bcl-2 and Mcl-1 with high affinity and disrupts Bcl-2/Bax and Mcl-1/Bak interactions (Zhang et al. 2011). In this case, the Mcl-1 antagonizing activity was questioned as it appeared that S1 uses an indirect mechanism (Noxa induction) to counteract Mcl-1 expression (Albershardt et al. 2011).

Also some of the synthetic nonpeptidic BH3 mimetics have been questioned for their specificity for antiapoptotic proteins. In several cases they were found to exert cytotoxic effects independently of their interaction with antiapoptotic proteins. The (−) enantiomer of the plant-derived gossypol polyphenol named AT-101 (Kitada et al. 2003) is able to displace the binding of BH3-only proteins from Bcl-XL and antagonize the activity of the latter (Kitada et al. 2003). Also, AT-101 overcomes stroma-mediated Mcl-1 induction and apoptosis protection in chronic lymphocytic leukemia (Balakrishnan et al. 2009). However, gossypol does not kill cells solely as a BH3 mimetic (van Delft et al. 2006; Albershardt et al. 2011). A few stabler, less toxic derivatives of gossypol are under investigation (e.g., TW37, ApoG2, and BI-97C1), but they do not seem to display high affinity for antiapoptotic proteins.

Screening a natural product library led to the design of the cycloprodigiosin-derivative small molecule GX15-070 (obatoclax mesylate). GX15-070 binds to all antiapoptotic proteins but, again, with rather low affinities. Nevertheless, it was able to release Bak from Mcl-1, to liberate Bim from Bcl-2 and Mcl-1 (Nguyen et al. 2007; Konopleva et al. 2008), and was the first putative BH3 mimetic to enter clinical trials in oncology (Schimmer et al. 2008). However, it was also demonstrated to activate apoptosis also by Bcl-2-independent pathways (Konopleva et al. 2008; Albershardt et al. 2011).

To date, the most studied and promising synthetic BH3 mimetic is ABT-737 (Oltersdorf et al. 2005). This small organic molecule was discovered by a method based on nuclear magnetic resonance screening and the structure–activity relationship to identify compounds that bind to the hydrophobic groove of Bcl-XL (Oltersdorf et al. 2005). It inhibits all antiapoptotic proteins, but Mcl-1, with a binding affinity markedly higher than all previously reported BH3 mimetics. ABT-737 was demonstrated to be a true Bad-like BH3 mimetic (Oltersdorf et al. 2005; van Delft et al. 2006). It exhibits apoptotic efficacy against several cell tumor cell lines and ex vivo tumor cells (Oltersdorf et al. 2005; Konopleva et al. 2006; Del Gaizo Moore et al. 2007; Kang et al. 2007), potentiates the activity of therapeutic agents (Kang et al. 2007; Cragg et al. 2009), and counteracts human tumor growth in mouse xenograft models (Konopleva et al. 2006). However, ABT-737, like its second-generation orally bioavailable compound ABT-263

(Tse et al. 2008), is effective only if Mcl-1 activity is impaired (Konopleva et al. 2006; van Delft et al. 2006), owing to its Bad-like BH3 mimetic structure. As an adjuvant to ABT-737 for killing tumors that overexpress both Bcl-XL and Mcl-1, a recent peptidic BH3 mimetic was proposed, BimS2A (Lee et al. 2008). It derives from the Bim BH3 domain modified to be highly selective for Mcl-1, and cannot be used as a single agent either, since it promotes cell death only when Bcl-XL is neutralized (Lee et al. 2008).

Of the putative or true BH3 mimetics, so far only three have entered clinical investigations, GX15-070, AT-101, and the orally administered version of ABT-737, navitoclax (ABT-263). Table 9.1 reports information on ongoing or completed clinical studies. According to the first data arising from completed phase I/II trials, both GX15-070 and AT-101 have limited therapeutic activity. Phase I/II studies of single-agent AT-101 showed no objective responses in men with castrate-resistant prostate cancer (Liu et al. 2009) nor in extensive-stage small cell lung cancer (Baggstrom et al. 2011). According to phase II combination studies, AT-101 did not show improved activity when used in combination with topotecan in relapsed and refractory small cell lung cancer (Heist et al. 2010) or with docetaxel in non-small-cell lung cancer (Ready et al. 2011).

Likewise, GX15-070 showed limited clinical activity in patients with relapsed or refractory Hodgkin lymphoma (Oki et al. 2012) and did not augment the antitumor activity of topotecan in relapsed small cell lung cancer (Paik et al. 2011).

It is not known whether these disappointing results depend on the fact that the compounds are not true BH3 mimetics. Nevertheless, other ongoing clinical trials (Table 9.1) might provide more encouraging results.

For ABT-263, promising results have been achieved so far. A first phase I dose-escalation study designed to assess the safety and antitumour activity of navitoclax in patients with lymphoid tumors and establish the drug's pharmacokinetic and pharmacodynamic profiles (Wilson et al. 2010) showed that navitoclax is safe and well tolerated, with dose-dependent thrombocytopenia (Wilson et al. 2010) and, in some cases, thrombocytopathy (Schoenwaelder et al. 2011) as the major adverse effects. Preliminary efficacy data from a phase I study in patients with small cell lung cancer and other solid tumors (Gandhi et al. 2011) and in patients with relapsed and refractory chronic lymphocytic leukemia were very encouraging (Roberts et al. 2012). Nevertheless, the final conclusions of these studies have to be awaited, particularly of those that foresee co-treatments of navitoclax with compounds able to downregulate Mcl-1. BH3 mimetics generally lack overall single-agent efficacy, and are mostly effective when used together with other compounds so that they can target the whole spectrum of Bcl-2 antiapoptotic proteins.

9.6 Concluding Remarks

Much investment has been devoted in the last few years to obtain mechanistic insights into the biology of apoptosis. BH3-only proteins are essential actors for the control of the mitochondrial apoptotic pathway, and compelling evidence supports their nomination as promising candidate therapeutic targets.

However, further investigations are needed to obtain mechanistic insights into the biological features of BH3 mimetic compounds and to assess whether they represent a safe and efficacious chemotherapeutic approach for tumor patients. Thus, the suitability of BH3 mimetics as target-specific antineoplastic therapeutic compounds in distinct malignant diseases has to be further explored, with particular regard to mimetics targeting selectively Mcl-1. Eagerly awaited additional studies will have to establish whether BH3 mimetics promote permeabilization of the MOM selectively in tumor cells, while preserving healthy cell survival, without the cost of widespread apoptosis of normal tissues.

In conclusion, we still have to discover if BH3 mimetics will meet the expectations of oncologists and provide a valuable addition to their armamentarium soon.

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Chapter 10
Signaling Pathways of MTA Family Proteins as Regulators of Cancer Progression and Metastasis

Yasushi Toh and Garth L. Nicolson

Abstract The metastasis-associated (*MTA*) gene family is a family of cancer-progression-related genes and their encoded products. The expression of *MTA1* messenger RNA or MTA1 protein has been repeatedly reported to correlate with various malignant properties, including prognosis in a wide range of human cancers. MTA proteins are involved in the nucleosome remodeling and histone deacetylation complex and function as transcriptional corepressors. In addition, tumor suppressor protein p53 is deacetylated and inactivated by both MTA1 and MTA2 proteins, leading to inhibition of growth arrest and apoptosis. Moreover, hypoxia-inducible factor 1α is also deacetylated and stabilized by MTA1 protein, resulting in stimulation of angiogenesis. MTA1 protein also functions as a coactivator of several genes related to cancer and inflammation. Recent studies clearly show that the MTA family has important roles in epithelial-mesenchymal transitions and DNA damage response. Thus, MTA proteins, especially MTA1, are a possible set of master coregulatory molecules involved in the carcinogenesis and progression of various malignant tumors. MTA proteins are proposed to be important new tools for clinical applications in cancer diagnosis and treatment.

Keywords Metastasis-associated gene 1 • MTA family • Chromatin remodeling • Histone deacetylation • Gene expression • Protein modification • Cancer progression • Metastasis • Epithelial–mesenchymal transitions • DNA damage response • Cancer growth

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Abbreviations

ARF	Alternative reading frame
ATM	Ataxia teleangiectasia mutated
ATR	Ataxia teleangiectasia mutated and Rad3-related protein
cDNA	Complementary DNA
COP1	Constitutive photomorphogenesis protein 1
DSB	Double-strand break
EIF5A2	Eukaryotic initiation factor 5A2
EMT	Epithelial–mesenchymal transitions
ER	Estrogen receptor
ERE	Estrogen-receptor-responsive element
GSK3β	Glycogen synthase kinase 3β
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HDAC	Histone deacetylase
HIF-1α	Hypoxia-inducible factor 1α
HMMR	Hyaluronan-mediated motility receptor
HRG	Heregulin β_1
HSF1	Heat shock factor 1
IHC	Immunohistochemistry
15-LOX-1	15-Lipoxygenase 1
LPS	Lipopolysaccharide
miR	microRNA
mRNA	Messenger RNA
MTA	Metastasis-associated
MyD88	Myeloid differentiation primary response gene 88
NuRD	Nucleosome remodeling and histone deacetylation
PolII	RNA polymerase II
RNAi	RNA interference
RT-PCR	Reverse-transcription polymerase chain reaction
shRNA	Short hairpin RNA
SUMO	Small ubiquitin-related modifier
TGF-β_1	Transforming growth factor β_1

10.1 Introduction

A number of cancer-related genes and molecules have been discovered in the last few decades. We identified a candidate metastasis-associated (*MTA*) gene that was abundantly overexpressed in highly metastatic rat mammary adenocarcinoma cell lines compared with poorly metastatic cell lines (Toh et al. 1994, 1995). When this gene was sequenced, it was revealed as a completely novel gene without any homologous or related genes in the database. The rat gene was named *mta1*

(metastasis-associated gene 1). A homologous gene was also expressed in human cancer cell lines (Toh et al. 1994), and its human complementary DNA (cDNA) counterpart, *MTA1*, was cloned by our group in 2000 (Nawa et al. 2000). Using surgically resected human tissues, we showed that high levels of *MTA1* mRNA expression were clinicopathologically correlated to the invasive and growth properties of gastrointestinal cancers, including esophageal, gastric, and colorectal cancers (Toh et al. 1997, 1999). Subsequently, many reports from independent research groups followed our observations and showed similar correlations between *MTA1* expression and the malignant potentials of human cancers (Toh and Nicolson 2009, 2011).

Several genes related to *MTA1* have now been identified, indicating *MTA1* is a member of a gene family, which we now call the "*MTA* family". Further studies on molecular biological and biochemical properties of the *MTA* family have shown that the gene products of the main members of the family (MTA1, MTA2, and MTA3 proteins) are tightly associated in a protein complex called the nucleosome remodeling and histone deacetylation (NuRD) complex, which has transcriptional regulatory functions via histone deacetylation and chromatin remodeling. Moreover, the NuRD complex exerts its deacetylating function with non-histone proteins, such as p53 and hypoxia-inducible factor 1α (HIF-1α). Furthermore, MTA1 protein possesses a transcriptional coactivator function (Li DQ et al. 2012). Thus, the *MTA* family has attracted widespread attention as one of the key groups of molecules that play an indispensable role in the genesis and progression of a wide variety of cancers (Kumar et al. 2003; Manavathi and Kumar 2007; Manavathi et al. 2007b; Toh and Nicolson 2009; Li DQ et al. 2012).

In 2009 we reviewed the clinical, pathological, and biological relevance of the *MTA* family with respect to carcinogenesis and cancer progression (Toh and Nicolson 2009). Here, we will examine the most recent advances in research on the *MTA* family, with special attention being paid to the significance of *MTA1* expression in human cancers and the important molecular mechanisms by which MTA proteins exert their functions.

10.2 The Expression of MTA Proteins in a Wide Variety of Human Cancers and Its Clinicopathological and Biological Relevance

10.2.1 Clinicopathological Relevance of Increased MTA1 Expression in Human Cancer Tissues

Using a reverse-transcription polymerase chain reaction (RT-PCR) method, we demonstrated that the higher expression of *MTA1* mRNA in surgically resected human gastric and colorectal cancer specimens compared with the paired normal counterpart tissues was significantly correlated to the depth of cancer invasion

Table 10.1 Clinicopathological implications of increased MTA1 expression in various human cancer tissues: reports after 2008

Type of cancer	Method	Clinicopathological implications	Reference
Breast cancer			
	IHC	higher LN mets. higher stage correl. with VEGF-A	(Sharma et al. 2011)
Gastrointestinal cancer			
Esophageal			
SCC	IHC	poorer prognosis (in node-negative cases)	(Li SH et al. 2009)
SCC	IHC	poorer prognosis correl. with VEGF-A higher LN mets.	(Li SH et al. 2012)
SCC	IHC	deeper wall invasion higher LN mets. poorer prognosis	(Song et al. 2012)
Barrett	IHC	higher expression in Barrett mucosa	(Miyatani et al. 2011)
Colorectal cancer	IHC	deeper wall invasion larger Tumor size higher LN mets. correl. with VEGF-C correl. with LVD	(Du et al. 2011)
	IHC	poorer prognosis (in combination with HDAC1)	(Higashijima et al. 2011)
Pancreatic endocrine tumor	IHC	malignant > benign WHO class Larger tumor size Higher mitotic rate	(Hofer et al. 2009)
Hepatocellular	SNP	higher recurrence rate	(Lee SH et al. 2012)
Non-small cell lung cancer	IHC	more advanced stage higher LN mets. poorer prognosis	(Zhu et al. 2010)
	IHC	poorer prognosis (in stage I cases)	(Yu et al. 2011)
	IHC	poorer prognosis correl. with MVD (in early stage cases)	(Li SH et al. 2011)
Ovarian cancer	IHC	more advanced stage poorer prognosis	(Prisco et al. 2012)
Head & Neck cancer			
Nasopharyngeal	IHC	poorer prognosis	(Li WF et al. 2012)

(continued)

Table 10.1 (continued)

Type of cancer	Method	Clinicopathological implications	Reference
Tonsil	IHC	higher LN mets.	(Park et al. 2011)

See our previous review concerning the reports before 2008 (Toh and Nicolson 2009)
SCC squamous cell carcinoma, *IHC* immunohistochemistry, *SNP* single nucleotide polymorphism
MVD microvessel density, *LN mets*. lymph node metastases, *LVD* lymph vessel density, *VEGF* vascular endothelial growth factor, *HDAC1* histone deacetylase 1

and lymph node metastasis (Toh et al. 1997). This study was the first to demonstrate the clinical relevance of *MTA1* expression with respect to the malignant potentials of human cancers. Subsequently, using a RT-PCR method, we found that human esophageal squamous cell cancers overexpressed *MTA1* mRNA. The cases in which *MTA1* mRNA was overexpressed showed significantly higher frequencies of adventitial invasion and lymph node metastasis and tended to have a higher rate of lymphatic involvement (Toh et al. 1999). By use of immunohistochemistry (IHC) we further examined the protein expression level of MTA1 in human esophageal squamous cell cancers and reconfirmed the results on *MTA1* expression obtained by RT-PCR (Toh et al. 2004). In this study, we also demonstrated that the overexpression of MTA1 protein was a predictor of poor prognosis after surgery.

Since the reports by us showing that the upregulation of *MTA1* gene expression was significantly correlated to the malignant properties of human cancers were published (Toh et al. 1997, 2004), many investigators have been documenting the expression levels of MTA family members, especially the MTA1 protein, in clinical samples from various human cancers. The reports before 2008 were summarized in our previous review (Toh and Nicolson 2009). Several subsequent studies have now been published, most of which have reinforced the previous information on the clinicopathological relationship between MTA1 protein overexpression and malignant potentials of various human cancers. Recent studies on this subject have been performed mainly using IHC owing to the commercial availability of MTA1 antibodies. The updated information on MTA1 protein expression in clinical samples after 2008 is summarized in Table 10.1.

10.2.2 Biological Relevance of MTA Proteins to Carcinogenesis and Cancer Progression

In addition to the clinicopathological evidence for the role of MTA1 protein mentioned already, the biological relevance of MTA proteins to carcinogenesis and cancer progression has been made much clearer by experiments that more directly tested the involvement of MTA1 protein in invasion and metastasis.

Direct evidence showing the association of MTA1 protein expression with the malignant properties of breast cancer was first obtained by Mazumdar et al. (2001). They demonstrated that forced overexpression of MTA1 protein in the breast cancer cell line MCF-7 was accompanied by enhancement of the ability of cells to invade an artificial matrix and to grow in an anchorage-independent manner. They also showed that the enhancement of MTA1 expression was associated with the interaction between MTA1 protein and histone deacetylase (HDAC), resulting in repression of transcription mediated by estrogen receptor (ER) α.

The study of Mazumdar et al. (2001) was extended by further experiments by the same group, where they showed direct in vivo evidence of the involvement of MTA1 protein in the carcinogenesis of breast cancer in an animal model (Bagheri-Yarmand et al. 2004; Singh and Kumar 2007). This group established transgenic mice that overexpressed MTA1 protein. They then found that the *MTA1*-transgenic mice showed inappropriate development of mammary glands, and the mice eventually developed hyperplastic nodules and mammary tumors, including mammary adenocarcinomas.

Recently, Jiang et al. (2011) demonstrated that short hairpin RNA (shRNA)-mediated silencing of *MTA1* in the ER-negative breast cancer cell line MDA-MB-231 resulted in reexpression of ERα and reduced protein levels of matrix metalloprotease 9 and cyclin D1. This, in turn, resulted in reduction of the invasiveness and proliferation of MDA-MB-231 cells. However, these changes seen by *MTA1* gene silencing were not observed in the ER-positive breast cancer cell line MCF-7.

Clinicopathological correlations with MTA1 protein overexpression in squamous cell carcinomas were reinforced by the experimental results of Mahoney et al. (2002). They transfected *MTA1* cDNA into immortalized human keratinocytes and clearly showed that forced expression of MTA1 contributed to several aspects of enhanced metastatic behavior in the anchorage-independent states of immortalized keratinocytes, including increased rates of migration, invasion, and survival. Furthermore, Qian et al. (2005) inhibited *MTA1* expression by RNA interference (RNAi) in a human esophageal squamous cell carcinoma cell line and showed significant inhibition of in vitro invasion and migration properties of the cancer cells.

Direct evidence showing the role of MTA1 protein in the progression of pancreatic cancer was provided by Hofer et al. (2004). They transfected *MTA1* cDNA into the pancreatic cell line PANC-1 and demonstrated that enhanced expression of MTA1 protein promoted the acquisition of an invasive and metastatic phenotype. It also enhanced the malignant potentials of pancreatic adenocarcinomas that were produced by modulation of the cytoskeleton via IQGAP1.

Results similar to those described above were reported in prostate cancers (Kai et al. 2011). MTA1 knockdown by RNAi inhibition of *MTA1* transcription significantly suppressed the in vitro invasion and angiogenic activities of prostate cancer cell lines with high expression levels of MTA1 protein, such as LNCaP, PC3, and DU145. Tumor formation and development in mouse xenografts was also inhibited in those cells. In addition, analysis of MTA1 expression using a human tissue microarray revealed a positive correlation between the intensity of immunostaining of MTA1 protein and the aggressiveness of prostate cancers.

Thus, the available evidence clearly demonstrates the biological relevance of MTA1 protein in the genesis and progression of many types of cancers.

10.3 Molecular Mechanisms of the MTA Family in Physiological and Pathological Conditions, Especially in Carcinogenesis and Cancer Progression

As mentioned already, by using different approaches in various laboratories that MTA1 protein overexpression closely correlated with carcinogenesis and cancer progression of a wide range of cancers originating in disparate organs and tissues. This strongly indicates that the MTA1 protein may be one of the more important molecules in cancer progression. Here, we introduce several of the important molecular mechanisms that have been recently clarified for the physiological and pathological functions of MTA proteins, especially those that are concerned with carcinogenesis and cancer progression. A schematic presentation of the molecular mechanisms of the MTA1 protein is shown in Fig. 10.1.

10.3.1 NuRD Complex and Transcriptional Repression

The first observations on the molecular and biochemical functions of MTA1 protein were obtained by four independent groups in 1998–1999 (Tong et al. 1998; Xue et al. 1998; Wade et al. 1999; Zhang et al. 1999; Bowen et al. 2004). In these

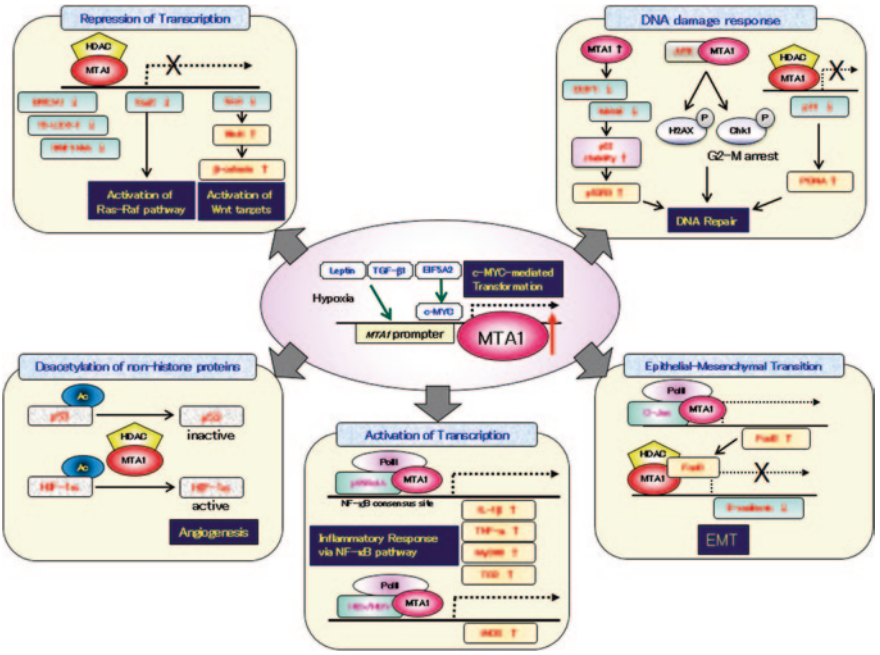

Fig. 10.1 The main molecular mechanisms of MTA1 protein in carcinogenesis and cancer progression

studies, two disparate chromatin-modifying activities, ATP-dependent nucleosome remodeling activity and histone deacetylation, were functionally and physically linked in the same protein complex. This complex has been named the nucleosome remodeling and histone deacetylation complex (NuRD complex), and it contains HDAC1, HDAC2, the histone-binding proteins RbAp46 and RbAp48, and the dermatomyositis-specific autoantigen Mi-2, which has been shown to have transcription repressing activity. Xue et al. (1998) reported that the MTA1 protein was found in the NuRD complex, where it had strong transcription repressing activity. Subsequently, Zhang et al. (1999) reported that a protein similar to MTA1 (named MTA2) was also a component of the NuRD complex and that MTA2 was highly expressed in rapidly dividing cells. Later, MTA3 was identified as an estrogen-inducible gene product that forms a distinct NuRD complex (Fujita et al. 2003). We also reported the physical interaction between MTA1 and HDAC1 (Toh et al. 2000).

Thus, the fundamental functions of the MTA family members appear to be exerted through a NuRD complex that has chromatin remodeling and histone deacetylating properties. The MTA/NuRD complex shows transcriptional repression activities (Fearon 2003; Kumar et al. 2003; Manavathi and Kumar 2007; Manavathi et al. 2007b; Singh and Kumar 2007). Although all MTA family proteins are found in NuRD complexes, these proteins form distinct NuRD complexes that are thought to target different sets of promoters (Bowen et al. 2004).

Although the involvement of MTA proteins in NuRD complexes suggested that such complexes might function in chromatin remodeling and histone deacetylation, a direct target of MTA proteins was first identified by Mazumdar et al. (2001). MTA1 was identified as a molecule induced by a growth factor, heregulin β_1 (HRG), which is a natural ligand of the human epidermal growth factor receptors HER3 and HER4 that can also transactivate HER2 (c-erbB-2) in human breast cancer cell lines. They showed that MTA1 directly interacted with the ligand-binding domain of ERα and that HRG stimulated the chromatin association of MTA1 and HDAC2 on an ER-responsive element (ERE) in the promoters of the estrogen-responsive genes, such as pS2 and c-myc. This may explain the phenomenon that activation of the HRG/HER2 pathway in ER-positive breast cancers results in the suppression of ERα functions, resulting in more invasive and aggressive phenotypes being observed in ER-negative breast cancers (Tang et al. 1996).

The repressive function of MTA1 protein on ERα is mediated through histone deacetylation by HDAC1 and HDAC2, suggesting that MTA1 has a potent corepressor function on the transactivation function of ERα through histone deacetylation. MTA2 protein has also been shown to physically interact with ERα and to repress its transactivating functions. Furthermore, overexpression of MTA2 protein rendered cells unresponsive to estrogen and suppressed estrogen-induced colony formation in breast cancer cells (Cui et al. 2006). Khaleque et al. (2008) showed that MTA1 bound to heat shock factor 1 (HSF1), the transcriptional activator of the heat shock genes, in vitro and in human breast carcinoma samples. They demonstrated that HSF1/MTA1 complex formation was strongly induced by HRG and that the complex was incorporated into the NuRD complex and participated in repression of estrogen-dependent transcription in breast cancer cells treated with HRG.

In addition, Molli et al. (2008) reported that MTA1/NuRD complexes negatively regulated BRCA1 transcription by physically associating with the ERE of the BRCA1 promoter in an ERα-dependent manner. This repressive effect of MTA1 protein on BRCA1 expression resulted in an abnormal centrosome number and chromosomal instability.

A short form of MTA1 protein was subsequently identified and named MTA1s (Kumar et al. 2002). MTA1s is a splice variant of MTA1 and contains an ER-binding motif (nuclear binding motif) without any nuclear localization signals at the C-terminus. This protein localizes in the cytoplasm, where it sequesters ERα, resulting in the prevention of ligand-induced nuclear translocation of ERα and stimulation of the malignant phenotype of breast cancer cells. This suggests that the regulation of the cellular localization of ERα by MTA1s may represent a mechanism for redirecting nuclear receptor signaling by nuclear exclusion. MTA1s has also been shown to associate with casein kinase $1\gamma_2$, which is an estrogen-responsive kinase (Mishra et al. 2004b).

More recently, many target genes where the MTA1/NuRD complex exerts its corepressor function have been identified. For example, 15-lipoxygenase 1 (15-LOX-1) is transcriptionally silenced in many cancer cells, and reactivation of its transcription restores apoptosis in these cells. Zuo et al. (2009) demonstrated that the knockdown of key components of NuRD such as MTA1 and HDAC1 proteins by small interfering RNA activated 15-LOX-1 transcription in SW480 and Caco-2 colon cancer cells. Using real-time polymerase chain reaction, they also showed that *MTA1* RNA expression in colon cancer tissues obtained from colorectal cancer patients was negatively related to 15-LOX-1 expression. It will be interesting to investigate whether the same or a similar mechanism exists in other types of cancers, such as esophageal, breast, and pancreatic cancers, where 15-LOX-1 expression is known to be downregulated.

Induction of MTA1 protein expression is also sufficient to transform Rat1 fibroblasts. This is caused by activation of the Ras–Raf pathway via transcriptional repression of $G_{\alpha i2}$, a negative regulator of Ras, by recruitment of the MTA1/HDAC complex to the $G_{\alpha i2}$ promoter. This suggests that MTA1 protein is a new class of regulator of the Ras pathway. In this situation, acetylation of MTA1 protein is crucial for its transforming activity (Ohshiro et al. 2010).

Marzook et al. (2012) showed that MTA1 protein drives tumor cell migration and invasion through transcriptional repression of the RING finger protein 144A. This RING finger protein has potential E3 ubiquitin ligase stimulating activity by recruitment of HDAC2 and CCAAT/enhancer-binding protein α corepressor complex to its promoter. This inverse correlation between MTA1 and RING finger protein 144A expression was also observed in publicly available breast cancer microarray data sets and the MCF10 breast cancer progression model system.

In 2010, an important new finding on MTA1 function was reported (Kumar et al. 2010a). MTA1 and MTA1s proteins were found to interact with *Six3* chromatin and repress its transcription. The protein product of the*Six3* gene is a direct HDAC-inhibitor-dependent repressor of *Wnt1* transcription. Kumar et al. (2010a) also that mammary glands from the *MTA1s/MTA1*$^{-/-}$ mice exhibited increased

recruitment of the Six3 corepressor complex to the *Wnt1* promoter, inhibiting the Wnt1 pathway in mammary glands. Consequently, MTA1 and MTA1s proteins were found to hyperactivate the Wnt1 pathway owing to increased expression of *Wnt1* transcription, indicating that MTA1 and MTA1s may be the important upstream activators of the Wnt1/β-catenin pathway.

In addition, MTA1s is known to form a complex with growth factor receptor binding protein 2–SOS in the cytoplasm, thus activating the Ras pathway and promoting ERK stimulation in breast cancer cells (Kumar et al. 2002). Kumar et al. (2002) further demonstrated a remarkable correlation between the levels of MTA1s and stimulation of the Wnt1 signaling components, which results in increased stability of β-catenin and stimulation of Wnt1 target genes in murine mammary epithelial and human breast cancer cells. This suggests a mechanistic role for the ERK/glycogen synthase kinase 3β (GSK3β)/β-catenin pathway in the stimulation of the Wnt1 target genes by MTA1s (Kumar et al. 2010b).

10.3.2 Deacetylation of Non-Histone Proteins by the NuRD Complex, Including the MTA Family

The protein targets for deacetylation by HDAC via NuRD complexes containing MTA proteins are not only chromatin histones but are also other non-histone proteins. The tumor suppressor gene product p53 was the first non-histone protein that was reported to be deacetylated by MTA-protein-containing NuRD complexes. Luo et al. (2000) found that the deacetylation of p53 was mediated by an HDAC1 complex containing MTA2 protein. This MTA2-associated NuRD complex interacted with p53 in vitro and in vivo and reduced significantly the steady-state levels of acetylated p53. Deacetylation of p53 results in an increase in its own degradation through MDM2 and thus a reduction in p53-dependent transcriptional activation. This eventually leads to the repression of the normal p53 functions that mediate cell growth arrest and apoptosis. The same phenomenon was observed between p53 and MTA1 proteins. HDAC1/MTA1 complexes possessed deacetylation activity against p53 protein in human non-small-cell carcinoma and human hepatoma cells, and the complexes were found to inhibit p53-induced apoptosis by attenuating the transactivation function of p53 (Moon et al. 2007).

In other studies, Kai et al. (2010) showed that resveratrol, a dietary compound from grapes that has anticancer properties, restored the p53 signaling pathway in prostate cancer cells. Resveratrol causes downregulation of the MTA1 protein and destabilizes MTA1/NuRD complexes, thus leading to acetylation and activation of the p53 protein and stimulation of p21 and Bax, which then can induce apoptosis.

Another important non-histone protein that is deacetylated by HDAC1/MTA1 complexes is HIF-1α-inducible factor, a key regulator of angiogenic factors (Yoo et al. 2006). The expression of MTA1 protein is strongly induced under hypoxic conditions in breast cancer cell lines, and MTA1 overexpression was found to enhance

the transcriptional activity and stability of HIF-1α protein. MTA1 protein physically binds to HIF-1α and deacetylates it. By increasing the expression of HDAC1, one can enhance the stabilization of HIF-1α. These results indicate evidence for positive cross talk between MTA1 and HIF-1α, mediated by HDAC1 recruitment. They also indicate the existence of a close connection between MTA1-associated metastasis and HIF-1α -induced tumor angiogenesis. Furthermore, Moon et al. (2006) showed that MTA1 protein increased the transcriptional activity of HIF-1α and the expression of vascular endothelial growth factor, a target molecule of HIF-1α. Conditioned medium collected from *MTA1* gene transfectants increased angiogenesis in vitro and in vivo.

A functional link between HIF-1α and MTA1 proteins has been demonstrated in clinical samples of pancreatic carcinomas. Using IHC and surgically resected pancreatic carcinomas, Miyake et al. (2008) examined the expression of HIF-1α, HDAC1, and MTA1 proteins and suggested that HIF-1α expression, which is associated with a poor prognosis in patients with pancreatic cancers, might be regulated by HDAC1/MTA1 complexes.

The potential contribution of MTA1 protein to tumor angiogenesis was also demonstrated in human breast cancers. Using IHC, Jang et al. (2006) examined MTA1 protein expression and intratumoral microvessel density in clinical breast cancer samples and showed that MTA1 expression was significantly correlated with higher tumor grade and higher tumor microvessel density. The relationship between MTA1 expression and microvessel density was also observed in hepatitis B virus (HBV)-associated hepatocellular carcinomas (HCC) (Ryu et al. 2008).

Recently, Yoo et al. (2008) experimentally demonstrated that the transactivator protein HBx of HBV strongly induced the expression of MTA1 and HDAC1, resulting in a physical link between these proteins and HIF-1α. This suggests that positive cross talk between HBx and the MTA1/HDAC1 complex occurs and may be important in stabilizing HIF-1α, which could, in turn, play a critical role in angiogenesis and metastasis of HBV-associated HCC (Yoo et al. 2008). As shown in Table 10.1, clinicopathological correlations between MTA1 expression and angiogenesis have been demonstrated in many other systems.

10.3.3 MTA1 Functions as a Coactivator in Addition to a Corepressor

Although the fundamental functions of MTA proteins are exerted via transcriptional repression by histone deacetylation, MTA1 also interacts with RNA polymerase II (PolII) and acts as a coactivator on several targets. Gururaj et al. (2006a, b) showed that breast cancer amplified sequence 3, a gene amplified and overexpressed in breast cancers, was a chromatin target of MTA1 and that the transcription of breast cancer amplified sequence 3 was stimulated by MTA1. This suggested that MTA1 has a transcriptional coactivator function in addition to a corepressor function. A similar finding was also suggested for mouse Mta2 protein (Matsusue et al. 2001).

In other studies, Pakala et al. (2010a) showed that MTA1 protein acted as a transcriptional coactivator of inflammatory cytokines in *Escherichia coli* lipopolysaccharide (LPS)-stimulated macrophages, whereas it acted as a corepressor in resting primary macrophages. LPS stimulates *MTA1* transcription via the NF-κB pathway, and then the MTA1/PolII/p65 RelA complex is recruited to the promoters of NF-κB target genes, such as interleukin-1α and tumor necrosis factor α. There it upregulates the transcription of these genes. In this same study they also showed that *MTA1*$^{-/-}$ mice were much more susceptible than control mice to septic shock induced by LPS, revealing that MTA1 protein protects mice from deregulated host inflammatory responses.

The transcription of the myeloid differentiation primary response gene 88 (*MyD88*) gene is also activated by LPS through recruitment of the MTA1/PolII/p65 RelA complex to the NF-κB consensus site in the *MyD88* promoter (Pakala et al. 2010b). MyD88 is one of the adaptor molecules of the Toll-like receptor family, and it eventually activates NF-κB transcription, leading to induction of proinflammatory cytokines. Furthermore, LPS stimulation in macrophages induces transcription of transglutaminase 2 in the same manner, aberrant activation of which has been linked with various inflammatory diseases and many types of cancers (Ghanta et al. 2011). All of these data suggest that MTA1 protein is associated with inflammatory responses as well as with cancer.

Bui-Nguyen et al. (2010b) revealed that the transactivator protein HBx of HBV stimulated the expression of *MTA1* but not *MTA2* or *MTA3* RNAs. Its underlying mechanism involves HBx targeting of NF-κB and the recruitment of an HBx/p65 complex to the NF-κB consensus motif in the *MTA1* promoter. Positive association between MTA1 and HBx, and MTA1 and NF-κB–p65 intensities in IHC was observed in HCC patients but not in non-HCC patients. Particularly, in HBx-positive HCC patients, there was a significant positive association between NF-κB–p65 and MTA1 intensity (Bui-Nguyen et al. 2010b).

MTA1 protein induced by HBx subsequently forms a protein complex with HBx, and this complex activates the transcription of inducible nitric oxide synthase in an NF-κB-dependent manner (Bui-Nguyen et al. 2010a). Nitric oxide has been implicated in the pathogenesis of inflammatory diseases, including HBV-associated HCC. Bui-Nguyen et al. (2010a) showed that HBx-mediated stimulation of MTA1 was paralleled by the suppression of microRNA (miR)-661. Depletion of MTA1 by suppressing *MTA1* RNA by either miR-661 or small interfering RNA in HBx-expressing cells severely impaired the ability of HBx to modulate the endogenous levels of inducible nitric oxide synthase and thus nitric oxide production.

Several other genes that have their transcription rates upregulated by MTA1 protein have been found. Li DQ et al. (2011) demonstrated that MTA1 protein activated the transcription of a tumor suppressor gene, alternative reading frame (ARF), by recruiting the transcription factor c-Jun and PolII onto the ARF promoter in a p53-independent manner. Importantly, ARF, in turn, inhibits MTA1 protein expression by blocking *MTA1* transcription or by affecting MTA1 protein stability. This indicates the presence of an autoregulatory feedback loop that regulates both MTA1 and ARF activities. Thus, downregulation of ARF by gene deletion, mutation, or promoter methylation can lead to unrestricted MTA1-mediated

cell proliferation and transformation, resulting in the absence of an "oncogenic checkpoint." Actually, low-ARF/high-*MTA1* transcription levels correlated well with reduced disease-specific survival in breast cancer patients harboring mutant p53 (Li DQ et al. 2011). MTA1/c-Jun/PolII coactivator complexes also stimulate the transcription of hyaluronan-mediated motility receptor (HMMR) gene in breast cancer cells. The protein product of the HMMR gene is a cell surface oncogenic protein, which is widely upregulated in human cancers and correlates well with cell motility and invasion. Indeed, MTA1 protein expression levels correlated with HMMR expression in human breast and prostate cancers during progression (Sankaran et al. 2012).

10.4 Roles of MTA Family Proteins in Epithelial–Mesenchymal Transitions

The MTA family plays an important role in epithelial–mesenchymal transitions (EMT). MTA3, the latest addition to the MTA family, was identified as an estrogen-dependent component of the Mi-2/NuRD transcriptional corepressor complex in breast epithelial cells (Fujita et al. 2003). The absence of MTA3 as well as the absence of ER results in aberrantly increased expression of the transcriptional repressor Snail, a master regulator of EMT. This increased expression of Snail results in reduction in the expression of the cell adhesion molecule E-cadherin and subsequent changes in epithelial architecture and invasive growth. The *MTA3* gene is a transcriptional target of ERα, and in the presence of estrogen, ERα can directly bind to the *MTA3* promoter at the SP1 site in close proximity to the ERE half-site. This results in stimulation of *MTA3* transcription (Fujita et al. 2004; Mishra et al. 2004a). Thus, MTA3 protein functions to maintain a differentiated, normal epithelial status in breast cells, which is in stark contrast to the effects of MTA1 and MTA1s proteins. Any potential upregulation of MTA1 can result in repression of MTA3 expression through repression of the ERα function, leading to upregulation of Snail, downregulation of E-cadherin, promotion of EMT, and consequently an increase in metastatic potential in breast cancer cells. In fact, Mishra et al. (2004a) reported that *MTA3* gene expression was regulated by the endogenous MTA1 protein, and the reduction of MTA1 protein expression resulted in a significant increase in both basal and estrogen-induced promoter activity of the *MTA3* gene. Furthermore, it was revealed that a transient forced expression of MTA1 led to loss of MTA3 protein in breast cancer cell lines (Fujita et al. 2004). Interestingly, the same phenomenon was also observed in an ovarian cancer cell line, in which MTA1 overexpression resulted in downregulation of E-cadherin and MTA3 expression and enhanced expression of Snail and Slug (Dannenmann et al. 2008).

The expression of MTA3 protein inhibits ductal branching in virgin and pregnant mammary glands in *MTA3*-transgenic mice (Zhang et al. 2006). This property is in contrast to what is found in *MTA1*-transgenic mice, where the inappropriate development of mammary glands results in the development of hyperplastic nodules and then

mammary tumors, including adenocarcinomas and lymphomas (Bagheri-Yarmand et al. 2004; Manavathi et al. 2007b).

MTA3 protein also represses Wnt4 transcription and Wnt4 secretion, inhibiting Wnt-target genes in mammary epithelial cells. This repression of Wnt4 transcription was found to be mediated through an MTA3/NuRD complex, which interacts with the Wnt4-containing chromatin in an HDAC-dependent manner (Zhang et al. 2006).

The transcription of *MTA1* is also related to EMT. Pakala et al. (2011) showed that the transcription of *MTA1* was stimulated by transforming growth factor β_1 (TGF-β_1) in epithelial cells and that *MTA1* transcriptional status was a determinant of TGF-β_1-induced EMT phenotypes. TGF-β_1 regulates the components of EMT via stimulating *MTA1* expression, which in turn induces FosB via an MTA1/PolII/AP-1 complex, resulting in repression of E-cadherin expression through recruitment of MTA1/HDAC2/FosB complexes to E-cadherin chromatin (Pakala et al. 2011).

Zhu et al. (2012) revealed that ectopic overexpression of a putative oncogene, eukaryotic initiation factor 5A2 (*EIF5A2*), in colorectal cancer cell lines enhanced cell motility and invasion in vitro and tumor formation in vivo. It also induced EMT, and Zhu et al. (2012) found that MTA1 protein was a potential downstream target of EIF5A2 in these cells. They further showed that the overexpression of EIF5A2 in colorectal cancer cells substantially enhanced the enrichment of c-myc on the promoter of the *MTA1* gene, and this *MTA1* upregulation by EIF5A2 was partly dependent on c-myc.

Yan et al. (2012) revealed that leptin could induce EMT in breast cancer cells. The leptin-induced EMT required β-catenin activation. Mechanistically, leptin stimulates phosphorylation of GSK3β via Akt activation, leading to increased accumulation of β-catenin. In addition, leptin increases MTA1 protein expression, resulting in upregulation of Wnt1 expression, which contributes to GSK3β phosphorylation and β-catenin activation. These data also suggested the presence of cross talk between leptin and MTA1/Wnt signaling in EMT of breast cancer cells.

10.5 Roles of MTA1 Protein in DNA Damage Response

The protein members of NuRD complexes, including MTA1 and MTA2 proteins, are co-immunoprecipitated with ataxia teleangiectasia mutated (ATM) and Rad3-related protein (ATR) (Schmidt and Schreiber 1999). ATR is a phosphatidylinositol kinase related enzyme that has been implicated in the response of human cells to multiple forms of DNA damage and may play a role in the DNA replication checkpoint. This fact suggests that MTA proteins may contribute in some way to the regulation of DNA checkpoints.

Although the report by Schmidt and Schreiber (1999) was published in 1999, it took another 10 years to determine the function of MTA1 in DNA damage responses (Li DQ and Kumar 2010; Li DQ et al. 2012). Li DQ et al. (2009b) discovered that MTA1 protein was required for optimum DNA double-strand break (DSB) repair after exposure to ionizing radiation. They showed that the stability of MTA1 protein was regulated by RING finger E3 ubiquitin protein ligase

constitutive photomorphogenesis protein 1 (COP1)-stimulated degradation via the ubiquitin–proteasome pathway. COP1 is rapidly degraded by ionizing radiation in an ATM-dependent manner, and the disruption of COP1-mediated proteolysis by ionizing radiation leads to stabilization of MTA1 protein. *MTA1*$^{-/-}$ mouse embryonic fibroblasts are hypersensitive to ionizing radiation exposure and exhibit decreased clonogenic survival compared with wild-type controls, suggesting that MTA1 protein is critical for efficient DSB repair. Because *MTA1*$^{-/-}$ mouse embryonic fibroblasts still contain MTA2 and MTA3 proteins, these findings are specific to MTA1 protein.

Li DQ et al. (2009a) examined the underlying mechanisms for the role of MTA1 protein in DNA DSB repair. They found that MTA1 controlled p53 stability by inhibiting its ubiquitination by E3 ubiquitin ligase mouse double minute 2 and COP1. Moreover, MTA1 protein competes with COP1 in binding to p53. These events lead to the regulation of p53-dependent transcription of p53R2, a direct p53 target gene for supplying nucleotides to repair damaged DNA. This indicates an inherent role of the MTA1/p53/p53R2 pathway in DNA damage responses in cancer cells. There is also a p53-independent function of MTA1 protein in DNA damage response. In this case, Li DQ et al. (2010b) showed that induced expression of MTA1 in p53$^{-/-}$ cells inhibited p21 promoter activity through the binding of HDAC2 and p21 binding to proliferating cell nuclear antigen, resulting in increased induction of histone 2A variant X foci and DNA DSB repair, thereby decreasing DNA damage sensitivity following treatment with ionizing radiation.

The function of MTA1 protein in the UV-induced DNA damage checkpoint pathway has also been studied in mammalian cells. Li DQ et al. (2010a) showed that MTA1 protein was stabilized in response to UV radiation in an ATR-dependent manner, which increased MTA1 binding to ATR, leading to activation by phosphorylation of many downstream substrates, such as checkpoint kinase 1 and histone 2A variant X. Depletion of MTA1 results in a defect in the G_2–M checkpoint and increases cellular sensitivity to UV-induced DNA damage.

Participation of MTA1 protein in DNA damage response has also been reported by another group. Chou et al. (2010) used a proteomics approach to find proteins involved in DNA repair and found that two components of the NuRD complex, CHD4 and MTA1, as well as polycomb group members are recruited to DNA lesions following exposure to UV radiation by poly(ADP-ribose) polymerase, which has been implicated in the recruitment of DNA repair factors to DNA lesions.

10.6 Roles of MTA1 Protein in Various Types of Stem Cells

Recently, new roles for MTA1 protein in various types of stem cells have been reported. Using rodent systems, Liang et al. (2008) showed that the transcription factors Nanog and Oct4, which regulate self-renewal and pluripotency of embryonic stem cells, interact with multiple repression complexes, including NuRD and

Sin3A, and that Mta1 was preferred among NuRD components for the Nanog and Oct4 complex to exert its function. They also showed that knockdown of Hdac1/2 and Mta1/2-containing Nanog- and Oct4-associated deacetylase led to increased expression of developmentally regulated genes and differentiation of embryonic stem cells.

Debeb et al. (2010) identified and characterized cancer cells with cancer-stem-cell-like features in 293T human embryonic kidney cells. These cells can be readily cultured and passaged as spheres in serum-free stem-cell-promoting culture conditions to form aggressive tumors with self-renewal potential after transplantation to mammary fad pads. Three-dimensional spheres generated from 293T cells show resistance to radiation, upregulating stem cell survival signals and increasing expression of mesenchymal genes and prometastatic genes, such as *MTA1*. Furthermore, Kumar et al. (2011) showed that inhibition of MTA1 expression by shRNA in immortalized human mesenchymal stem cells affects their proliferation and osteogenic differentiation.

10.7 Other Important Physiological Functions of MTA Proteins

Since MTA proteins are also expressed in normal cells and tissues, although at lower levels than found in cancer cells, it is important to understand the physiological functions and underlying mechanisms of action of MTA proteins in normal cells. Also, it is important to understand the pathological functions of MTA proteins in cancer cells.

The reported physiological roles of MTA proteins are as follows:

1. MTA1 protein is thought to play a crucial role in postnatal testis development and spermatogenesis (Li W et al. 2007a, b). MTA1 protein can apparently serve as a transient protector of primary spermatocytes against heat-stress-induced apoptosis. Therefore, MTA1 protein might act as a novel coregulator of p53 in maintenance of cellular integrity during an early phase after hyperthermal stimulation in testicular germ cells (Li W et al. 2011).
2. The expression level of the *MTA1* gene decreases in mouse brain in an age-dependent manner, which influences the estrogen-mediated signaling pathways during aging (Thakur and Ghosh 2009). MTA1 protein functions as an upstream coactivator of tyrosine hydrozylase in neural cells with PolII and DJ1 (Parkinson disease 7), which is a rate-limiting enzyme for dopamine synthesis (Reddy et al. 2011). Furthermore, in rodents a NuRD complex including Mta2 is required for peripheral nerve myelination (Hung et al. 2012).
3. MTA1 protein is a direct stimulator of rhodpsin expression (Manavathi et al. 2007a).
4. MTA1 protein stimulates hepatic proliferation in vivo and hepatocyte differentiation in vitro (Li W et al. 2008).

5. The zinc-finger and C-terminal domains of MTA proteins, including MTA1, MTA2, and MTA3, are required for FOG-2-mediated transcriptional repression via the NuRD complex in cardiac morphogenesis (Roche et al. 2008).
6. An Mta3/NuRD complex is essential for the initiation of primitive hematopoiesis in vertebrate embryos (Li X et al. 2009).
7. The MTA1 homologues egl-27 and egr-1 in *Caenorhabditis elegans* are related to embryonic patterning (Solari et al. 1999; Chen and Han 2001). NuRD complexes, including those containing egr-1, antagonize vulval development in *C. elegans*, which is induced by the Ras signal transduction pathway (Solari and Ahringer 2000).

10.8 Regulatory Mechanisms for MTA1 Expression and Function

It will undoubtedly become important to clarify the regulatory mechanisms that govern the expression and functions of MTA proteins, especially MTA1 protein.

An important finding for the MTA family is the relationship between MTA1 protein and c-MYC oncoprotein. By expression profiling, Zhang et al. (2005) identified MTA1 protein as a target of c-MYC protein in primary human cancer cells. In this case they showed that c-MYC oncoprotein binds to the genomic *MTA1* locus and recruits transcriptional coactivators. They also presented data suggesting that the MTA1 protein in NuRD complexes was one of the first downstream targets of c-MYC function. This was essential for the transformation potential of c-MYC, because reduction of MTA1 expression by an shRNA blocked the ability of c-MYC to transform mammalian cells.

Most recently, Lee MH et al. (2012) demonstrated that p53 transrepressed MTA1 expression via p53-binding elements located in the *MTA1* promoter and that poly(ADP-ribose)ylation of p53 by poly(ADP-ribose) polymerase 1 had an important function in the p53-mediated repression of MTA1 expression.

MicroRNAs (miRs) have been identified as posttranscriptional modifiers of target gene regulation, and they control the expression of gene products important in cancer progression. Two studies have reported on regulation of MTA1 expression by miRs. Reddy et al. (2009) showed that miR-661, a target of the transcription factor CCAAT/enhancer-binding protein α, which is downregulated during cancer progression, inhibited MTA1 expression. They also found that introduction of miR-661 inhibited the motility, invasiveness, anchorage-dependent growth, and tumorigenicity of invasive breast cancer cells by downregulation of MTA1. Zhou et al. (2012) noted that miR-30c negatively regulated the proliferation, migration, and invasion of endometrial cancer cells by binding to 3'-untranslated regions of *MTA1*. Thus, miRs may be developed as novel therapeutic agents for downregulating the expression of *MTA1*.

Recently, Ohshiro et al. (2010) revealed that inducing high levels of MTA1 protein is sufficient to transform Rat1 fibroblasts. In this case, the transforming

potential of MTA1 was dependent on its acetylation at Lys-626, leading to activation of the Ras–Raf pathway via transcriptional repression of $G_{\alpha i2}$, a negative regulator of Ras, by recruitment of the MTA1/HDAC complex to the $G_{\alpha i2}$ promoter.

MTA1 protein contains a functional small ubiquitin-related modifier (SUMO) consensus site and a SUMO-interacting motif at its C terminus. SUMOylation at Lys-509 located within the SUMO consensus site and at the SUMO-interacting motif synergistically regulates the corepressor activity of MTA1 protein (Cong et al. 2011).

10.9 Conclusions and Future Directions

This review has focused on the clinical and biological significance of the *MTA* gene family, paying particular attention to the cellular protein products and their relevance to carcinogenesis and cancer progression (invasion and metastasis). The fundamental activities of MTA proteins are thought to be their actions as transcriptional corepressors that function through histone deacetylation in NuRD complexes, which contain chromatin remodeling and histone deacetylating molecules. Repression of ERα transactivation functions by members of the MTA family, and in particular MTA1 protein, is mediated through deacetylation of ERE chromatin containing the ER-responsive genes. This has been one of the most extensively investigated activities, and the data clearly demonstrate that MTA1 protein expression results in tumor formation and progression. This has been most extensively investigated in mammary tissues, where MTA1 renders breast cancer cells phenotypically more aggressive. In addition, MTA proteins have other activities, such as the ability to deacetylate non-histone proteins. For example, the tumor suppressor protein p53 is deacetylated and inactivated by both MTA1 and MTA2, resulting in inhibition of growth arrest and apoptosis. Another activity mediates blood vessel formation, where HIF-1α is deacetylated and stabilized by MTA1, leading to angiogenesis.

Considering the many reports showing the clinical relevance of the expression of *MTA1* mRNA and its encoded MTA1 protein in a wide variety of human cancers (Toh and Nicolson 2009) as well as definitive studies showing the molecular and biochemical mechanisms of MTA protein activities (Li DQ et al. 2012), it is likely that MTA proteins, especially MTA1, represent master coregulatory molecules involved in the carcinogenesis and progression of various malignant tumors. It also strongly and clearly suggests the possibility that the MTA1 protein (or its gene) could be an excellent molecular target for cancer therapy as well as its use in cancer diagnosis/prognosis. Although studies are not yet available that show the clinical efficacy of targeting MTA proteins, several experiments have shown that MTA1 protein (or its gene) could be a molecular target for cancer therapy.

The first studies that suggested the possibility of targeting the *MTA1* gene or MTA1 protein were reported by Nawa et al. (2000) and Nicolson et al. (2003). They used antisense phosphorothioate oligonucleotides against *MTA1* mRNA

and found a growth inhibitory effect on human metastatic breast cancer cell lines. Since these reports, others have shown that inhibition of MTA1 expression, mainly by RNAi techniques, can result in inhibition of the malignant phenotypes of various cancers, as mentioned already. Inhibition of MTA1 expression or function could enhance the chemosensitivity of cancer cells by restoring the tumor suppressor function of p53, or it may inhibit tumor angiogenesis by destabilizing the angiogenesis-promoting function of HIF-1α. Moreover, inhibitors of MTA proteins may cooperate with HDAC inhibitors, which are now expected to be a new class of anticancer agents.

Another MTA family member, the MTA1s protein, may also be a useful target in the treatment of malignancies, such as breast cancers. MTA1s functions as a repressor of ERα transcriptional activity by binding and sequestering ERα in the cytoplasm (Kumar et al. 2002). MTA1s has a unique C-terminal 33 amino acid region containing a nuclear receptor box motif that mediates the interaction of MTA1s and ERα. Singh et al. (2006) showed that the MTA1s peptide containing this motif could effectively repress the ERα transactivation function, altering estrogen-induced proliferation and anchorage-independent growth of human breast cancer cells. Using an animal model, they also showed the effect of MTA1s peptide in blocking the tumor progression of MCF-7 cells overexpressing ERα.

Although it is an intracellular protein, there is a possibility that MTA1 protein could be a target for immunotherapy. In a review of a model for immunotherapy using a virus vector, disabled infectious single-cycle herpes simplex virus, Assudani et al. (2006) proposed that MTA1 might be a promising new antigen for tumor rejection, because it is greatly overexpressed in many different tumors and is only expressed at much lower levels in normal tissues. Their initial studies demonstrated the presence of immunogenic MHC class I restricted peptides of MTA1. Furthermore, MTA1 was identified as a SEREX antigen, and hence it is likely to be capable of inducing a T-cell response in cancer patients (Li G et al. 2004).

MTA1 will also be clinically useful for the prediction of the malignant potentials of various human cancers, such as esophageal, gastric, and colorectal cancers. Thus, evaluating the expression levels of MTA proteins in individual cases of various cancers may provide clinicians with important clues for prognosis and possible anticancer therapies.

In conclusion, the MTA proteins, especially MTA1, may be excellent new candidates for therapy and diagnosis/prognosis of human cancers. For these reasons, the MTA family should be intensively studied for possible clinical applications.

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Chapter 11
Signalling Pathways of β-Catenin/JNK in Carcinogenesis

Eliana Kalakouti, Roya Babaei-Jadidi and Abdolrahman S. Nateri

Abstract Tight control of signalling/transcriptional activity ensures that the correct balance between gene expression changes regulating the cell cycle, DNA repair, developmental potential and fate determination is maintained by different cells. Activation of the Wnt signalling pathway stimulates growth and mediates developmental and carcinogenic signalling between cells. In fact, a Wnt pathway mutation, such as loss of the adenomatous polyposis coli gene, is sufficient to give rise to a tumor that grows without limit. This suggests that the mutation, through ectopically activating the Wnt pathway, may also switch on associated pathways; Notch, Eph/ephrin, bone morphogenetic protein, Hedgehog and mitogen-activated protein kinase, as observed in the $Apc^{Min/+}$ mouse model of intestinal carcinogenesis. Wnt signalling can also activate non-canonical, β-catenin-independent pathways, including activation of the c-Jun N-terminal kinase (JNK). JNK activation, which can occur via many types of cellular stress or extracellular signals, plays an essential role in organogenesis during mouse development by regulating cell proliferation, survival and apoptosis. JNK activation is also involved in messenger RNA stabilization, cell migration, cytoskeleton integrity and carcinogenesis. However, the links between the β-catenin and JNK pathways are poorly understood, and the identities of the downstream targets/effectors of Wnt/JNK remain largely unknown. Moreover, little is known about the effect of phosphorylation by JNK on the functions of the possible common targets between these two pathways. This chapter discusses the relations between the β-catenin and JNK pathways and their identifies component genes involved in carcinogenesis.

Keywords Wnt signalling pathway • β-catenin/JNK pathways • Adenomatous polyposis coli gene • Transcriptional activity • Cancer

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Abbreviations

AP1	Activator protein 1
APC	Adenomatous polyposis coli
GSK3β	Glycogen synthase kinase 3β
HMG	High mobility group
JNK	c-Jun N-terminal kinase
LEF	Lymphoid enhancer factor
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
STAT	Signal transducer and activator of transcription
TCF	T cell factor

11.1 The Pathway to Cancer

Cancer is a disease rooted in the *genes* mediated by *proteins* and manifested in the *cell* as 'unregulated cell growth' guilty of causing disease. The interplay between transcription (genes), signalling (proteins) and carcinogenesis (cell) is currently in the spotlight of cancer research, and *'pathways'* appear to govern the chain of events leading to disease. Widely accepted evidence suggests that cancer behaviour is better predicted using pathway principles rather than the individual genes. Even though there are fewer of the former than the latter (Vogelstein and Kinzler 2004), the number belies the complexity. Increasing evidence reveals that signalling components are often shared between individual pathways and that individual components talk to each other through multiple mechanisms (McNeill and Woodgett 2010). The realization that individual pathways are not subject to the traditionally assumed fidelity of their signalling components crucially impedes the potential to dissect the exact mechanisms responsible for carcinogenesis.

Normal cell development relies on signalling pathways mediating the communication within and between cells, thereby coordinating cell proliferation and homeostasis. Wnt signalling is a signal transduction pathway implicated in embryonic development, cell proliferation, stem cell maintenance and adult tissue homeostasis. The c-Jun N-terminal kinase (JNK) pathway is another pathway required for embryogenic morphogenesis. It is implicated in multiple physiological processes, including regulation of cell proliferation and apoptosis (Roger 2000). Accumulation of defects in the aforementioned regulatory circuits will compromise cell harmony and predispose to the transformation of normal human cells into highly malignant derivatives (Hanahan and Weinberg 2000).

11.2 Wnt Pathway: The Classic Literature

The Wnt signal transduction pathway, named after its most upstream players, the Wnts, has a fundamental contribution in the regulation of cell behaviour (cell proliferation, apoptosis and differentiation) and consequently cell fate (Cadigan and Nusse 1997; Peifer and Polakis 2000; Logan and Nusse 2004; Nusse 2005). It is not surprising, therefore, that the pathway is actively implicated in embryogenic morphogenesis, maintenance of cellular homeostasis and the self-renewing ability of adult tissues. Despite the great contribution in normal development, the pathway is normally inactive in adult unstimulated cells and will lead to tumor formation when aberrantly or inappropriately activated. Any mutational subversion in the pathway holds the potential of deregulated cell behaviour leading to diseases such as cancer (Giles et al. 2003).

The mammalian Wnt gene family of signalling molecules consists of 19 glycoproteins secreted in a paracrine fashion by various cell types (Cadigan and Nusse 1997). Collectively, the Wnt ligands are best known as the key players of three distinct signalling cascades:

1. The so-called canonical developmental cascade, the effects of which are mediated by the transcriptional programme of the β-catenin/T cell factor (TCF)/lymphoid enhancer factor (LEF) complex (Fig. 11.1).
2. The 'β-catenin independent' cascade, which is mainly involved in planar cell polarity signalling. An important role for JNK in the non-canonical Wnt signalling pathway has been established (Fig. 11.1).
3. The Wnt-dependent calcium/protein kinase C dependent pathway.

11.2.1 β-Catenin and the Wnt

β-Catenin, encoded by the human *CTNNB1* gene, constitutes an integral component of the canonical Wnt signalling cascade. Being a member of the Armadillo repeat protein superfamily, it features a central stretch of 12 imperfect Armadillo repeats (labeled R1-R12), including a core TCF interaction region. TCFs are members of the high mobility group (HMG) box protein family and form the predominant partner of β-catenin (Giese et al. 1992; Love et al. 1995). TCFs bind to DNA via an HMG domain and to β-catenin via a short stretch of amino acids to form a bipartite transcription factor. Stabilized β-catenin enters the nucleus and associates with TCF/LEF transcription factors, assisting their binding to Wnt-responsive elements. In this way TCFs form an absolute prerequisite for expression of β-catenin target genes (Graham et al. 2000; Huber and Weis 2001). Even though TCFs lack transactivation function, in β-catenin, the N-terminal and in particular the C-terminal regions that flank the Armadillo repeat domain exhibit transcriptional activation function (Behrens et al. 1996; Huber et al. 1996).

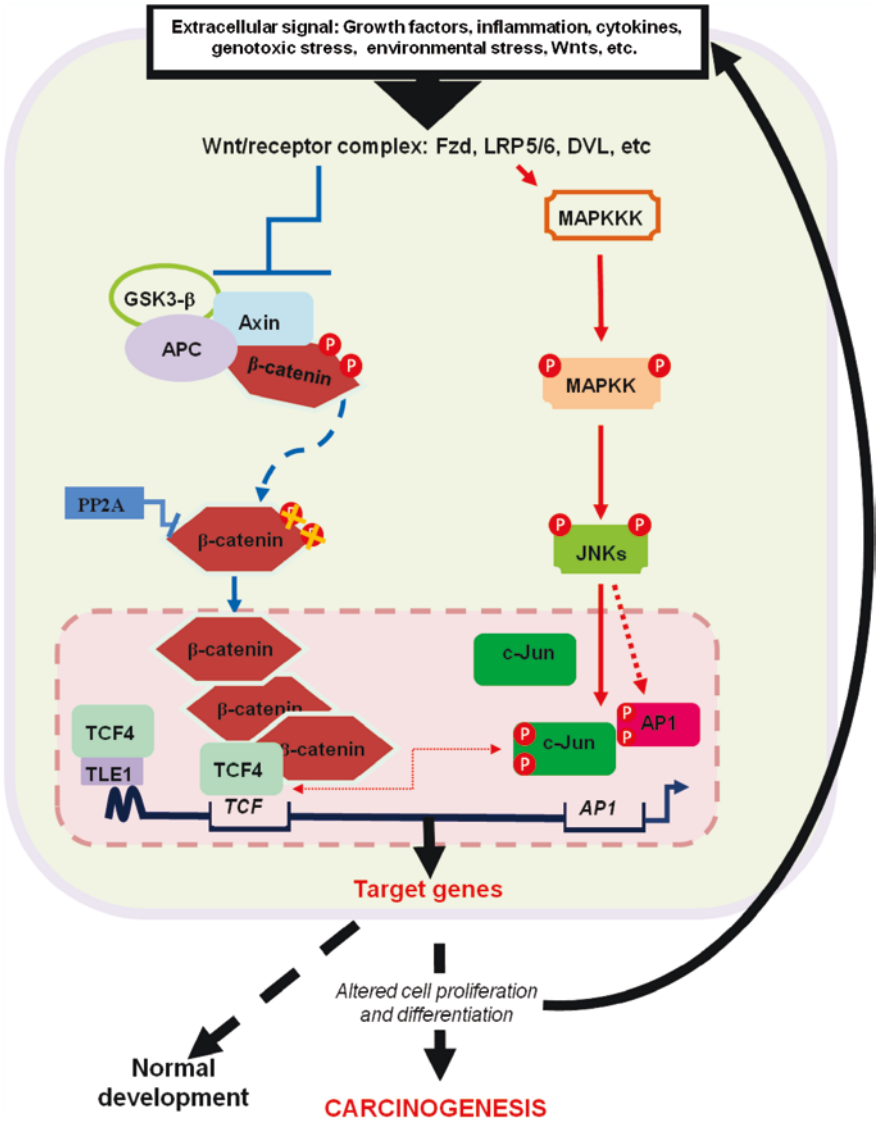

Fig. 11.1 Representation of the canonical Wnt/β-catenin and non-canonical Wnt/c-Jun N-terminal kinase (*JNK*) pathway components showing that these pathways cooperate physiologically in tumorigenesis. Molecular interactions between downstream key regulators such as c-Jun and T cell factor 4 (*TCF4*) cooperatively enhance the transcriptional activities of promoters containing activator protein 1 (*AP1*) and T cell factor (*TCF*) binding sites. The resultant proteins can promote a positive feedback loop for many Wnt target genes, with reported roles in cancer, in particular human colorectal tumors. *APC* adenomatous polyposis coli, *DVL* Dishevelled, *Fzd* Frizzled, *GSK-3β* glycogen synthase kinase 3β, *LRP* low density lipoprotein receptor related protein, *MAPKK* mitogen-activated protein kinase kinase, *MAPKKK* mitogen-activated protein kinase kinase kinase, *PP2A* protein phosphatase 2A

In absence of the Wnt ligand, β-catenin is present in an unstimulated steady-state cytosolic environment in which it is subject to constant degradation by the 'axin destruction complex'. As the name suggests, the complex is assembled over the scaffold protein axin, which binds β-catenin and comprises the tumor suppressor adenomatous polyposis coli (APC) and the serine/threonine kinases glycogen synthase kinase 3β (GSK3β) and casein kinase 1α (Hart et al. 1998; Liu et al. 2002; Clevers 2006; Klaus and Birchmeier 2008). The multiprotein complex cooperates to phosphorylate β-catenin, which is subsequently recognized by the E3 ligase β-transducin repeat containing protein for ubiquitination and degradation by the 26S proteosome (Kitagawa et al. 1999; Winston et al. 1999). The continual elimination of β-catenin prevents the protein from reaching the nucleus.

In the absence of nuclear β-catenin, the Wnt target genes are instead bound and repressed by the TCF/LEF family of transcription factors, which recruit the Groucho transcriptional repressor to prevent any Wnt-independent expression (Brannon et al. 1997). Groucho (TLE1 in mammals) functions with histone deacetylases to stimulate the compression of local chromatin and inhibit transcription (Cavallo et al. 1998; Roose et al. 1998; Brantjes et al. 2001; Courey and Jia 2001).

11.2.2 Wnt/β-Catenin and Cancer

In the presence of the Wnt ligand, the canonical Wnt signalling pathway is activated as shown in Fig. 11.1. Signal transmission is initiated when the Wnt ligand binds the seven-pass transmembrane domain receptor Frizzled and the low density lipoprotein receptor related protein 5 or 6 co-receptors (Bhanot et al. 1996; Tamai et al. 2000; Wehrli et al. 2000; Mao et al. 2001). The cytoplasmic protein Dishevelled is then recruited to the receptor complex, causing subsequent sequestration of axin from the degradation complex (Bilic et al. 2007; Huang and He 2008). The APC–axin complex is therefore disrupted, along with its ability to mark β-catenin for destruction (Polakis 2000; Seidensticker and Behrens 2000). By these means β-catenin escapes degradation and is stabilized in its hyperphosphorylated form to associate with the DNA-binding transcription factors of the TCF/LEF family in the nucleus. In this case, nuclear β-catenin competes with Groucho for TCF binding, thus replacing the repressor with an activation scaffold (Fig. 11.1). Under physiological activation, the pathway mediates embryonic development and adult homeostasis, regulating the expression of genes governing growth, survival and cell fate. In cancer, inappropriate activation of the pathway recklessly drives the expression of the aforementioned genes, resulting in uncontrolled cell proliferation (Behrens and Lustig 2004; Paul 2007).

Phosphorylation sites are essential for efficient degradation of β-catenin, and even single mutations in any of the sites will lead to β-catenin escaping destruction and its stabilization (Hinck et al. 1994; Munemitsu et al. 1995). In pathologic conditions β-catenin escapes degradation when its phosphorylation sites are mutated, or when components of the destruction complex are defective, such as

APC. Mutations in β-catenin rendering the pathway constitutively active were first identified in sporadic colorectal cancers (Korinek et al. 1997; Morin et al. 1997) and melanoma (Rubinfeld et al. 1997). Furthermore, the discovery that mutations in β-catenin prevented its negative regulation by APC (Korinek et al. 1997; Morin et al. 1997; Rubinfeld et al. 1997) fortified the oncogenic relationship of the two in the Wnt pathway. *APC* and *CTNNB1* mutations are mutually exclusive, consistent with the notion that mutation of either gene has more or less the same effect on β-catenin stability and β-catenin/TCF4 transactivation.

11.3 Mitogen-Activated Protein Kinase: The Wider Family

The evolutionarily conserved mitogen-activated protein kinase (MAPK) family consists of proline-directed serine/threonine kinases categorized into three main subgroups: the extracellular-signal-regulated kinases, the JNKs and the p38 kinases. The MAPK signalling pathway is activated in response to mitogenic factors and is one of the most widespread mechanisms of eukaryotic cell regulation. It relays, amplifies and integrates signals from a diverse range of extracellular stimuli, by mediating communication to the nucleus, altering gene expression and consequently changing cell behaviour (Fig. 11.1).

A plethora of signals can activate the mammalian MAPK pathway, which will always assemble over a three-tiered phosphorylation cascade as shown in Fig. 11.1. On stimulation, MAPK kinase kinases phosphorylate and activate MAPK kinases. The latter phosphorylate and activate MAPKs (MAPK) (Roger 2000) in their conserved Thr-X-Tyr dual-phosphorylation motif. MAPKs in turn dictate the intracellular response by phosphorylation and activation of downstream effectors, among them transcription factors. Expression of the appropriate genes then dictates the fate of biological processes such as proliferation, differentiation, cell survival, development, inflammatory response and apoptosis.

11.3.1 JNK in Physiology and Pathology

JNKs are also known as stress-activated protein knases, and exemplify one subgroup of the MAPKs. As their alternative name suggests, JNKs have well-documented functions in cellular response to stress (cytokines, UV irradiation and environmental stress), but are also critical regulators of normal physiology (Weston and Davis 2002). In the human genome, three loci encode JNK1, JNK2 and JNK3, all of which are alternatively spliced to create at least ten isoforms (Gupta et al. 1996). JNK1 and JNK2 are found ubiquitously, whereas the pattern of expression for JNK3 is restricted to the brain and to a lesser extent to the heart and testis. Collectively, JNKs were reported to exhibit important functions in embryonic morphogenesis, cell proliferation, cell death, involvement in the

mammalian immune system, T lymphocyte differentiation, control of insulin signalling, regulation of neuronal functions and epithelial sheet migration (Karin and Gallagher 2005).

The importance of JNKs stems from the fact that they are capable of operating within a diverse range of molecular targets, phosphorylating and regulating a wide array of signalling partners such as ATF2, Elk-1, signal transducer and activator of transcription (STAT) 3, c-Myc, Akt, IRS-I and members of the Bcl-2 family (Weston and Davis 2002; Karin and Gallagher 2005; Bogoyevitch and Kobe 2006; Wagner and Nebreda 2009; Chen 2012). Given the multitude of cellular processes in which they participate and the cell context and stimulus-specific nature within which they operate, it is not surprising that the action of JNK is not exclusive to a single behaviour. JNK may act to mediate stress responses, promoting perpetuation of defective genetic material and thus cellular transformation. Alternatively, it can provide a protective response against stress by promoting death of stressed cells. Widely accepted evidence implicates JNK in apoptosis on cellular stress.

The contribution of JNK in cancer can be attributed to the capacity of its substrates. A major, and possibly the best known, nuclear target of the JNKs is c-Jun, a member of the activator protein 1 (AP1) transcription factor family. JNK binds the N-terminal domain of the AP1 component, c-Jun proto-oncoprotein, phosphorylating it on serines 63 and 73 and threonines 91 and 93 (Pulverer et al. 1991; Smeal et al. 1991, 1992). Such phosphorylation is mainly responsible for the AP1 transcriptional activity, with a lesser contribution from other phospho-AP1-related factors (ATF2, JunB, JunD) (Smeal et al. 1992; Minden et al. 1994; Roger 2000; Kyriakis and Avruch 2001). Inductions of AP1-dependent target genes are implicated in the regulation of many biological processes, such as cell proliferation, cell death, inflammation, DNA repair and transformation.

11.3.2 JNK in Cancer: A Controversial Identity

Much attention has focused on the opposing roles of JNK in the regulation of cell death and survival (Potapova et al. 2000). A paradigm of JNK's role in apoptosis is in its use in therapeutic agents such as paclitaxel, a mitotic inhibitor used in cancer chemotherapy, and vinblastine, an antimicrotubule drug used in certain kinds of cancer. These agents use the property of JNK to initiate apoptosis to discontinue tumor growth (Mamay et al. 2003; Wang et al. 1998). Further, JNK-deficient primary fibroblasts were found to be resistant to UV-induced apoptosis owing to inefficient release of cytochrome *c* (Tournier et al. 2000).

Despite its proven involvement in the apoptotic response to stress, the JNK signal transduction pathway does not have exclusively proapoptotic behaviour, and its behaviour can even be contradictory. Human BT-474 breast carcinoma cells were reported to be refractory to treatment with the DNA-damaging agent cisplatin owing to enhanced cell survival in a JNK-dependent manner (Hayakawa et al.

2004). Likewise, JNK inhibition induced apoptosis in multiple cancer cell lines in vitro and slowed DU145 xenograft growth (Engelberg 2004). JNK-mediated augmented survival in response to stress could result in tumor progression and treatment resistance. Isoform-specific functions may also explain some of the varied and indeed contradictory effects attributed to JNK proteins and add to the complexity of the behaviour of JNK (Gupta et al. 1996).

11.3.3 JNK Signalling: A Positive Regulator of Carcinogenesis

In a signal transduction cascade, any component which can positively regulate the developmental pathway can be considered to be a potential oncogene (proto-oncogene). This is because the aberrant or inappropriate activation of such a component has the potential to recklessly drive the pathway and confer proliferative tendencies on the cell. Research in certain tumor types revealed JNKs as components of 'oncogenic' pathways by demonstrating that several oncogenes, such as *Ras*, can induce JNK activity (Smeal et al. 1991; Dérijard et al. 1994; Eferl and Wagner 2003). Examples of oncoproteins implicated in the transformation of several established cell lines in a JNK-dependent manner are insulin-like growth factor 1 in MCF-7 breast cancer cells (Monno et al. 2000), the RalGDs (a guanine nucleotide exchange factor that activates Ral) in skin tumor cells (González-García et al. 2005) and the leukemogenic oncogene *BCR-ABL* in haematopoietic cells (Hess et al. 2002). It is most likely that the response of JNK signalling to the aforementioned oncoproteins promotes enhanced cell survival and provides positive regulation of carcinogenesis through the JNK target genes. Likewise, experiments using murine c-Jun revealed that mutated c-Jun (with altered JNK phosphoacceptor sites) was unable to convey the JNK signal, and was therefore unable to cooperate in malignant transformation (Smeal et al. 1991; Behrens et al. 2000).

What is more, several loss-of-function studies support the contribution of JNK to transformation (Roger 2000). Studies using antisense oligonucleotides demonstrate that JNK inhibition is adequate to cause growth arrest or apoptosis in tumor cells (Potapova et al. 1997, 2000). Comparably, when multiple myeloma and breast cancer cell lines were treated with JNK inhibitors and antisense oligonucleotides, growth arrest was reported (Hideshima et al. 2003, Mingo-Sion et al. 2004). Regarding the involvement of JNK in gastric, hepatic and lung carcinogenesis, lack of JNK1 resulted in decreased cell proliferation (Shibata et al. 2008), impaired tumorigenesis (Sakurai et al. 2006) and reduced incidence of tobacco-smoke-induced lung tumors (Takahashi et al. 2010) respectively.

Details regarding the mechanism of JNK-mediated carcinogenesis are still unclear. A report on mediators of JNK-dependent carcinogenesis follows. The examples listed are taken from different JNK studies which have revealed JNK

operates within a diverse range of molecular targets, regulating in an array of signalling partners through which it can exemplify its carcinogenic nature:

1. Data regarding human melanoma revealed upregulation of JNK due to a constitutively active extracellular-signal-regulated kinase. Consequent stabilization of c-Jun increased transcription of target genes, including RACK1 and cyclin D, which further enhanced JNK activity, enforcing a feed-forward mechanism of the JNK/c-Jun pathway (Lopez-Bergami et al. 2007).
2. Furthermore, JNK was implicated in androgen-independent prostate cancer, in which it was found to mediate leptin-stimulated androgen-independent cancer cell proliferation via STAT3 and Akt.
3. The JNK pathway was also reported to contribute to cellular transformation by downregulating tumor suppressors such as p53 or nuclear receptors in lung cancer cells.
4. A distinct investigation on the importance of JNK phosphorylation on retinoic acid receptor α pointed to JNK as a key mediator of aberrant retinoid signalling in lung cancer cells, conferring chemoresistance (Srinivas et al. 2005).
5. In colon carcinogenesis, the induction of JNK activity resulted in direct phosphorylatation of Smad2/3 and increased the progression and extension of cancer (Yamagata et al. 2005).
6. In an investigation to determine the function of c-Jun N-terminal phosphorylation by JNK during oncogenic transformation, Ras-induced skin tumor and c-Fos-induced osteosarcoma were impaired in mice lacking c-Jun N-terminal phosphorylation. Further, activated c-Jun was shown to contribute to early stages of carcinogen-induced hepatocellular carcinoma by antagonizing p53 (Behrens et al. 2000).
7. In the HT-1080 fibrosarcoma cell line, the JNK/c-Jun pathway, DNA topoisomerase I and epidermal growth factor receptor were found to cooperate in the positive regulation of HT-1080 cell proliferation (Mialon et al. 2005).

11.3.4 The Flip Side of the Coin: JNK as a Negative Regulator of Carcinogenesis

In contrast to expectations, studies implicating JNK in antitumorigenic behaviour have also been reported, suggesting a potential function of JNK as a tumor suppressor in vivo. JNK deficiency was shown to cause profound increases in the number and growth of Ras-induced tumor nodules in vivo (Kennedy et al. 2003) and caused spontaneous intestinal tumors associated with downregulation of p21 in intestinal epithelial cells (Tong et al. 2007). More recently JNK activity was found to negatively regulate β-catenin signalling through the GSK3β pathway, suggesting alterations in β-catenin to be the likely culprits involved in intestinal tumor formation in *JNK1*$^{-/-}$ mice (Hu et al. 2008).

Investigations into the contribution of JNK1 in mice embryonic fibroblasts–UVA-induced skin carcinogenesis revealed that lack of JNK1 and the consequent inability to phosphorylate myelin transcription factor 1 suppressed caspase-3 cleavage and DNA fragmentation, which would otherwise lead to apoptosis. *JNK1*$^{-/-}$ mice were found to develop more UVA-induced papillomas than either their JNK1 wild-type counterparts or JNK2-deficient mice. The presence of JNK1 was therefore found to be essential in UVA-induced apoptosis and prevention of skin carcinogenesis (Choi et al. 2009).

11.3.5 JNK Isoenzymes: Conflicting Views

Data from JNK1- and JNK2-deficient mice suggest that the two isoenzymes perform distinct and even opposing functions in cellular regulation, acting as positive and negative regulators of the latter via differential regulation of c-Jun. Evidence implies that JNK1 is the major isoform responsible for c-Jun N-terminal phosphorylation, at serines 63 and 73, whereas JNK2 may be a negative regulator of this activity (Sabapathy et al. 2004). Investigations performed on soft tissues of childhood sarcoma revealed that silencing of JNK1 but not JNK2 repressed the growth of these tumor cells, indicating that JNK1 is proliferative, whereas JNK2 might be proapoptotic (Durbin et al. 2009a, b).

JNK2-deficient mice were shown to exhibit a proliferative advantage relative to their wild-type counterparts, owing to enhanced phosphorylation and stabilization of c-Jun (Sabapathy et al. 2004; Sabapathy and Wagner 2004). Direct evidence for the involvement of JNK2 in promoting skin cancer was obtained with *JNK2*$^{-/-}$ knockout mice in which consequent suppression of 12-*O*-tetradecanoylphorbol-13-acetate induced skin carcinogenesis (Chen et al. 2001). This effect was correlated with the inability of the mice to induce AP1 DNA-binding activity.

In contrast to the JNK2-deficient-type behaviour, JNK1-deficient mice revealed increased incidence of carcinoma, number of carcinomas per mouse and rate of tumor development compared with their wild-type counterparts (She et al. 2002). The most compelling evidence for the role of JNK1 in cancer initiation is from studies of hepatocellular carcinoma in which 50 % of the hepatocellular carcinoma samples exhibited higher activation of JNK1, but not JNK2, and JNK1 activation was associated with poorer prognosis (Hui et al. 2008; Chang et al. 2009a, b).

Resolving the confusion and improving the understanding of JNK's role in the cause of cancer will be critical for cancer therapy. It will provide answers to questions regarding whether the therapeutic effort should seek to inhibit or perhaps activate the JNKs. Currently, inhibition of c-Jun N-terminal phosphorylation is a novel therapeutic strategy to inhibit tumor growth in vivo (Manning and Davis 2003; Karin and Gallagher 2005). Small-molecule JNK inhibitors were proposed to be useful in cancer therapy on the basis of their ability to inhibit the induction of DNA repair genes in cells treated with cisplatin or other DNA-damaging drugs (Potapova et al. 2001). At a time when biology provides prognostic information

critically impacting clinical oncology, delineation of JNK activity and the clinical prospect this entails could prove promising to the new era of cancer therapeutics and targeted chemotherapy.

11.3.6 The Theory of Compensatory Proliferation

Chen (2012) suggests that the likely link bridging the contradictory functions of JNK in apoptosis and carcinogenesis is a compensatory proliferation of neighbouring cells in response to the JNK-mediated apoptotic cells (Fig. 11.2). On cellular stress, activation of JNK causes the release of Wnt and bone morphogenetic protein glycoproteins and IL-6, which in turn act in the neighbouring cells to activate the Wnt and Janus kinase/STAT pathways, respectively (Wu et al. 2010). Studies in *Drosophila* (Ryoo et al. 2004; Morata et al. 2011) and animal disease models (Maeda et al. 2005; Sakurai et al. 2006) have provided compelling evidence for such a compensatory mechanism (Fig. 11.2). It could therefore be plausible to hypothesize that human cancers could be a result of the compensatory overgrowth of stem cells, or transdifferentiation of non-stem cells into cancer stem cells in response to the JNK-stimulated compensatory signals. Evasion of apoptosis is a hallmark of cancer.

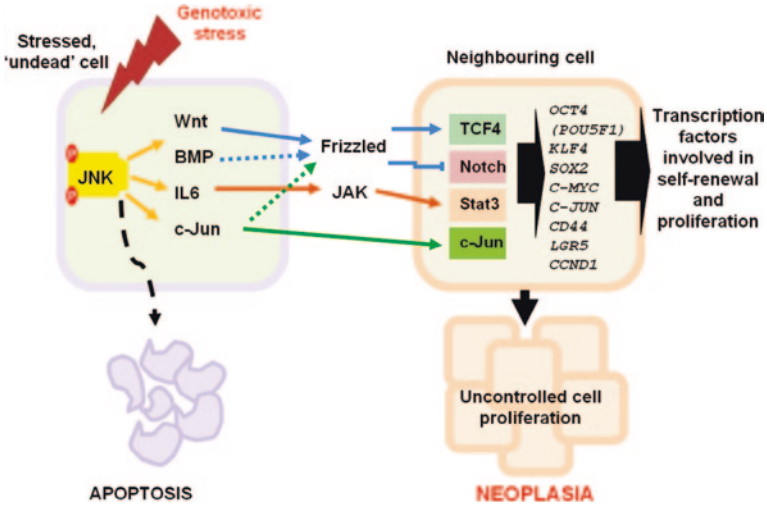

Fig. 11.2 Representation of the theory of compensatory proliferation demonstrating the link between the contradictory functions of JNK. JNK activation on cellular stress causes the release of Wnt and bone morphogenetic protein (*BMP*) glycoproteins and IL-6, which in turn act as compensatory signals in the neighbouring cells to activate the Wnt and Janus kinase (*JAK*)/signal transducer and activator of transcription pathways, respectively. *CCND1* cyclin D1, *KLF4* Krüppel-like factor 4, *LGR5* leucine-rich-repeat-containing G-protein-coupled receptor 5, *OCT4* octamer-binding transcription factor 4, *SOX2* sex determining region Y box 2, *STAT*3 signal transducer and activator of transcription 3

11.4 β-Catenin/TCF4/JNK/c-Jun: Partners in Crime

Nateri et al. (2005) reported that β-catenin acts synergistically with JNK to exacerbate the Wnt-mediated route to cancer, demonstrating for the first time an association between the two main culprits involved in neoplasia. In vivo and in vitro studies confirmed that phosphorylation of c-Jun by JNK enhanced the ability of c-Jun to interact with the HMG-box transcription factor TCF4, forming a ternary complex of c-Jun, TCF4 and β-catenin. This binds to the c-Jun promoter of target genes, enhancing the transcriptional activation of potential oncogenes, including c-Jun itself. The findings of Nateri et al. point to a mechanism whereby the interaction between TCF4 and phospho-c-Jun controls the transcriptional activation of c-Jun by recruiting β-catenin.

When β-catenin was knocked down, a marked reduction in the transcriptional activation of c-Jun by the TCF/c-Jun complex was observed, highlighting the essential involvement of β-catenin in the activation of the complex. To further support the lethal cooperation, it was noted that repression of c-Jun in experiments with defective/absent expression of c-Jun rendered β-catenin signalling markedly less able to induce tumor formation. Mutation in c-Jun greatly reduced the burden of tumorigenesis in $Apc^{Min/+}$ mice and the absence of c-Jun, and therefore the inability to form the TCF/c-Jun/β-catenin complex, delayed intestinal tumorigenesis and prolonged lifespan in mice (Raftopoulou 2005; Saadeddin et al. 2009).

Several Wnt target genes, including Wnts themselves, matrix metalloproteinases (MMPs), c-*myc*, cyclin D1, c-*jun*, *TCF4* and *Cd44*, are transcriptionally regulated through β-catenin/JNK pathways (Table 11.1) (Tetsu and McCormick 1999; Jochum et al. 2001; Eferl and Wagner 2003; Hayakawa et al. 2004; Hwang et al. 2005; Le Floch et al. 2005; Nateri et al. 2005; Gerdes et al. 2006; Igaki et al. 2006). Chromatin immunoprecipitation analysis showed that binding of c-Jun and β-catenin/TCF4 to the c-*jun* promoter is dependent on JNK activity (Nateri

Table 11.1 c-Jun N-terminal kinase (*JNK*) and β-catenin pathways cooperatively enhance transcriptional products of several oncogenes

β-Catenin/JNK target genes	References
c-*myc*	Yochum et al. (2008)
c-*jun*	Nateri et al. (2005)
Cyclin D1	Toualbi et al. (2007)
Mmp7	Crawford et al. (2001)
Mmp26	Marchenko et al. (2004)
Osteopontin	El-Tanani et al. (2004)
Cd44	Van der Flier et al. (2007), Nateri et al. (2005)
Wnt2	Le Floch et al. (2005)
Lgr5	Van der Flier et al. (2007), Aguilera et al. (2011)

Their promoters are contained in both the activator protein 1 (AP1) and the T cell factor DNA-binding sites and are found to be transcriptionally induced in cancers.

et al. 2005). Cyclin D1 is another important target gene through which c-Jun/AP1 controls proliferation (Wisdom et al. 1999). Similarly, the expression of cyclin D1 is regulated by both JNK and Wnt signalling (Tetsu and McCormick 1999; Wulf et al. 2001), and therefore AP1/β-catenin/TCF4 cooperation is directly involved in the transcriptional induction of cyclin D1. CD44 within the intestinal stem cell niche is both a c-Jun and a TCF4 target gene (Munoz et al. 2012; Nateri et al. 2005; Sabates-Bellver et al. 2007; Van der Flier et al. 2007). More recently, Wnt target gene *Lgr5*, the putative intestinal stem cell marker, has been identified as a target gene controlled by the c-Jun/JNK pathway (Aguilera et al. 2011) (Table 11.1).

Furthermore, Gan et al.(2008) reported that binding of c-Jun, TCF4, β-catenin and Dishevelled is required for full c-Myc expression. Moreover, association of c-Jun, β-catenin and TCF4, specifically with the downstream enhancer, triggers c-Myc transcription in human HCT116 colorectal carcinoma cells after mitogen stimulation (Yochum et al. 2008). Most notably, the use of a genome-wide chromatin immunoprecipitation on-chip analysis identified that TCF4 (*Tcf7l2*) is transcriptionally regulated through activated JNK signalling (Hayakawa et al. 2004) with three of the AP1/c-Jun binding sites, at base pairs −996 to −833 relative to the TATA box, of the *Tcf7l2* gene. We have shown TCF4/c-Jun co-localization in adenomas derived from $Apc^{Min/+}$ mice and microarray data suggest a significant similarity in TCF4 transactivation in response to mutations in APC and/or β-catenin genes (Sancho et al. 2009).

In mammary epithelia, the scenario seems to be different: El-Tanani et al. (2004) described that β-catenin/LEF-1 and c-Jun cooperate with the Ets transcription factor family in regulation of osteopontin transcription, which plays a key role in neoplastic transformation, metastasis (Weber 2001) and the prognosis of breast cancer (Patani et al. 2008). It was concluded that the presence of these transcription factors in human breast cancer is responsible for the overexpression of osteopontin (El-Tanani et al. 2004). Similarly, for AP1, the Ets transcription factor PEA3 synergizes with β-catenin/Lef-1 in the upregulation of the MMP matrilysin (MMP7) in intestinal tumors (Crawford et al. 1999, 2001), itself predominantly expressed in the cells of gastrointestinal, breast and lung carcinomas (McDonnell et al. 1991). Furthermore, Rivat et al. (2003) corroborated that Src-mediated activation of the human *MMP7* promoter requires the activation of AP1 signalling and a cooperative interaction between c-Jun and LEF-1 transcription factors (Rivat et al. 2003). Moreover, β-catenin, TCF/LEF, Ras and c-Jun interact with, and synergistically activate, the *MMP26* promoter (Marchenko et al. 2004). MMP26 is involved in hyperplastic and malignant endometrium (Pilka et al. 2004), and is overexpressed in skin cancer (Ahokas et al. 2005). Immunohistochemical analysis of human colorectal tumors showed nuclear expression of c-Jun, TCF4 and β-catenin (Takeda et al. 2008). However, induction of some of these target genes may require additional factors after prolonged tumor suppressor mutation (Babaei-Jadidi et al. 2011; Phelps et al. 2009).

11.5 Concluding Remarks

The cross talk between the pathways implicating β-catenin and JNK exemplify the emerging principles of signalling, and demonstrate the use of shared components between previously thought distinct paths. The finding that JNK/c-Jun and β-catenin/TCF4 cooperate along with the theory of JNK-compensatory proliferation activating the β-catenin-mediated Wnt pathway has important implications for understanding carcinogenesis and cancer therapeutics. Further, it illustrates the substantial amount of pathway integration that can occur at the level of gene regulation through the assembly and disassembly of transcriptional complexes (McNeill and Woodgett 2010). Such connivance among signalling factors is crucial in elucidating the tumorigenic behaviour of mutated cells.

Recognizing the complexity of genetic pathways is of outmost importance, especially when it comes to the major culprits involved in malignancy such as JNK and β-catenin. It is essential to appreciate that cancer behaviour is no longer considered to be a matter of individual genes alone. Leading the pathway to cancer is the interplay between key genes in transcription and signalling and recognizing this will allow a whole new era of cancer therapeutics to come into play.

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Chapter 12
The Regulation of the JNK Cascade and Programmed Cell Death by NF-κB: Mechanisms and Functions

Jason Bennett, Marta Moretti, Anil K. Thotakura, Laura Tornatore and Guido Franzoso

Abstract The nuclear factor κB (NF-κB) family is an evolutionarily conserved family of transcription factors that play a central role in immune and inflammatory responses. They also play a pivotal role in cell survival, whereby activation of NF-κB antagonizes programmed cell death induced by tumor necrosis factor receptors and other cell death signals. The prosurvival function of NF-κB has been implicated in a wide range of biological processes, including the development and homeostasis of the immune system and liver. It has also been implicated in the pathogenesis of numerous diseases, including cancer, chronic inflammation, and certain hereditary disorders. The protective activity of NF-κB can also hamper tumor cell killing inflicted by radiation or chemotherapeutic drugs, thereby promoting resistance to cancer treatments. This prosurvival activity of NF-κB involves the suppression of sustained c-Jun N-terminal kinase (JNK) activation and of the accumulation of cytotoxic reactive oxygen species. NF-κB mediates this function by inducing the transcription of target genes, whose products inhibit the JNK signaling pathway and suppress accumulation of reactive oxygen species through their antioxidant functions. The development of specific inhibitors that target the critical downstream NF-κB-regulated genes that promote survival in cancer and other diseases potentially holds a key to developing specific and effective therapeutic strategies to combat these disorders.

Keywords NF-κB • Programmed cell death • JNK • Reactive oxygen species • Cancer • Gadd45β • Inflammation

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Abbreviations

AP-1	Activator protein 1
ASK1	Apoptosis-signal-regulating kinase 1
BHA	Butylated hydroxyanisole
CAC	Colitis-associated cancer
cFLIP	Cellular FLICE-inhibitory protein
cIAP	Cellular inhibitor of apoptosis
CK2	Casein kinase 2
ConA	Concavalin A
FADD	Fas-associated death domain
FHC	Ferritin heavy chain
Gadd	Growth arrest and DNA damage inducible
HCC	Hepatocellular carcinoma
IAP	Inhibitor of apoptosis
IκB	Inhibitor of nuclear factor κB
IKK	Inhibitor of nuclear factor κB kinase
JNK	c-Jun N-terminal kinase
LUBAC	Linear ubiquitin chain assembly complex
MAP2K	Mitogen-activated protein kinase kinase
MAP3K	Mitogen-activated protein kinase kinase kinase
MAPK	Mitogen-activated protein kinase
MDR2	Multidrug resistance 2
MEF	Mouse embryonic fibroblast
MKP	Mitogen-activated protein kinase phosphatase
Mn-SOD	Manganese superoxide dismutase
NEMO	Nuclear factor κB essential modulator
NF-κB	Nuclear factor κB
PCD	Programmed cell death
RIP	Receptor-interacting protein
ROS	Reactive oxygen species
Smac	Second mitochondria-derived activator of caspases
TAK1	Transforming growth factor β activated kinase 1
TNF	Tumor necrosis factor
TNF-R1	Type 1 tumor necrosis factor α receptor
TNF-R2	Type 2 tumor necrosis factor α receptor
TRADD	Tumor necrosis factor receptor associated death domain
TRAF	Tumor necrosis factor receptor associated factor
XIAP	X-chromosome-linked inhibitor of apoptosis

12.1 Introduction

Programmed cell death (PCD) is a form of cellular suicide that plays a central role in various processes in animal physiology, including normal cell turnover and tissue homeostasis, as well as proper development and functioning of the immune system in order to eliminate defective or potentially dangerous cells (Rathmell and Thompson 2002; Agostini et al. 2011; Munoz-Pinedo 2012). Signaling pathways that govern cell death are of critical importance as inappropriate PCD (either too little or too much) is a major contributing factor in many human diseases (Rathmell and Thompson 2002; Agostini et al. 2011; Munoz-Pinedo 2012). Exaggerated cell death, for instance, is associated with neurodegenerative diseases, ischemic damage, and immunodeficiency, whereas at the other end of the spectrum, autoimmune diseases and cancer are linked to reduced cell death with concomitant increased cell survival (Rathmell and Thompson 2002; Agostini et al. 2011; Munoz-Pinedo 2012). Inducible regulation of gene expression allows organisms to adapt to environmental, mechanical, chemical, and microbiological stresses which could potentially lead to cell death. Since its discovery almost a quarter of a century ago, nuclear factor κB (NF-κB) has served as a model for inducible transcription factors (Oeckinghaus and Ghosh 2009). NF-κB plays its most important and evolutionarily conserved role in the innate and adaptive immune systems; however, it also acts broadly to influence gene expression events that impact cell survival, differentiation, and proliferation (Oeckinghaus and Ghosh 2009; Hayden and Ghosh 2012). The ability of NF-κB to suppress PCD is of major biomedical interest since the promotion of cell survival by NF-κB is a fundamental contributing factor in tumorigenesis, chemoresistance in cancer, and autoimmune diseases (Bubici et al. 2004; Luo et al. 2005; Papa et al. 2006). Advances over the last decade in the understanding of how NF-κB controls PCD have led to the discovery that the NF-κB-mediated antagonism of PCD involves the suppression of the c-Jun N-terminal (JNK) mitogen-activated protein kinase (MAPK) cascade and reactive oxygen species (ROS) (Bubici et al. 2004, 2006; Papa et al. 2006; Morgan and Liu 2011). This antagonism of PCD is mediated by a specialized subset of NF-κB target genes (discussed herein). Currently, there is a great deal of interest in developing drugs that target the products of these NF-κB-induced genes, with the aim of allowing selective inhibition of the prosurvival action of NF-κB, without compromising the capacity of NF-κB to serve in immunity, inflammation, and tissue development.

12.2 The NF-κB Pathway

Transcription factors of the NF-κB family are best known for their central role in immunity, inflammation, and oncogenesis (Oeckinghaus and Ghosh 2009; DiDonato et al. 2012; Hayden and Ghosh 2012). The mammalian NF-κB family consists of five members: RelA (p65), RelB, c-Rel, p50/NF-κB1, and p52/NF-κB2

(Oeckinghaus and Ghosh 2009; Oeckinghaus et al. 2011; DiDonato et al. 2012; Hayden and Ghosh 2012). All members of this family share a structurally conserved Rel homology domain, 300 amino acids in length at their N terminus, which mediates nuclear localization, DNA binding, dimerization with other NF-κB subunits, and interaction with inhibitors of NF-κB (IκBs) (Oeckinghaus and Ghosh 2009; Oeckinghaus et al. 2011; DiDonato et al. 2012; Hayden and Ghosh 2012). NF-κB family members are divided into two subfamilies based on divergence at their C terminus. The RelA (p65), RelB, and c-Rel proteins all contain C-terminal transactivation domains, which strongly activate transcription from NF-κB-binding sites within regulatory regions of target genes (Oeckinghaus and Ghosh 2009; Oeckinghaus et al. 2011; DiDonato et al. 2012; Hayden and Ghosh 2012). The proteins p50 and p52, which are generated from proteolytic cleavage of the precursor proteins p105 and p100, respectively, lack the transactivation domain but are still capable of binding to NF-κB-consensus DNA sites (Oeckinghaus and Ghosh 2009; Oeckinghaus et al. 2011; DiDonato et al. 2012; Hayden and Ghosh 2012). Although NF-κB is composed of numerous heterodimers or homodimers of various members, the most abundant and ubiquitous dimer in cells is p50(NF-κB1)/RelA(p65). Inducible NF-κB activation depends on the phosphorylation-induced proteosomal degradation of IκBs (IκBα, IκBβ, IκBγ, IκBε), which sequester inactive NF-κB dimers in the cytoplasm of unstimulated cells (Oeckinghaus and Ghosh 2009; Oeckinghaus et al. 2011; DiDonato et al. 2012; Hayden and Ghosh 2012). Most of the NF-κB-inducing signaling pathways converge on the IκB kinase (IKK) complex, which is responsible for IκB phosphorylation, thereby allowing the liberated NF-κB to enter the nucleus and regulate transcription of a variety of target genes encoding many immunoregulatory, inflammatory mediators and inhibitors of apoptosis (Oeckinghaus and Ghosh 2009; Oeckinghaus et al. 2011; DiDonato et al. 2012; Hayden and Ghosh 2012). Through the induction of these distinct sets of target genes, NF-κB coordinates innate and adaptive immunity, inflammation, cell differentiation, and cell survival (Oeckinghaus and Ghosh 2009; Oeckinghaus et al. 2011; DiDonato et al. 2012; Hayden and Ghosh 2012).

There are two signaling pathways that are primarily responsible for NF-κB activation, and which have been described as the classical or canonical pathway, and the alterative or noncanonical pathway (Oeckinghaus and Ghosh 2009; Oeckinghaus et al. 2011; Sun 2011; DiDonato et al. 2012; Hayden and Ghosh 2012). The IKK complex is composed of three subunits: the highly homologous catalytic subunits IKKα and IKKβ, and the nonenzymatic regulatory component, IKKγ/NF-κB essential modulator (NEMO) (Oeckinghaus and Ghosh 2009; Oeckinghaus et al. 2011; Sun 2011; DiDonato et al. 2012; Hayden and Ghosh 2012). Despite the structural similarities between IKKα and IKKβ, most stimuli that lead to NF-κB activation, including type 1 tumor necrosis factor (TNF) α receptor (TNF-R1), rely on IKKβ activity, and indeed IKKβ-deficient mice and cells exhibit defective TNFα-induced NF-κB activation (Oeckinghaus and Ghosh 2009; Oeckinghaus et al. 2011; Sun 2011; DiDonato et al. 2012; Hayden and Ghosh 2012). The classical pathway is triggered in response to stress, microbial products, and proinflammatory cytokines, resulting in the IKKβ-dependent

nuclear translocation of p50/RelA and p50/c-Rel dimers, and is involved primarily in the acute responses to infection and injury (Oeckinghaus and Ghosh 2009; Oeckinghaus et al. 2011; Sun 2011; DiDonato et al. 2012; Hayden and Ghosh 2012). The alternative pathway, which is generally activated by specific members of the TNF cytokine family such as lymphotoxin β, B cell activating factor, and the CD40 ligand, results in the specific activation of p52/RelB dimers (Oeckinghaus and Ghosh 2009; Oeckinghaus et al. 2011; Sun 2011; DiDonato et al. 2012; Hayden and Ghosh 2012). Unlike the classical pathway, which is dependent on IKKβ and IKKγ activity, the alternative pathway depends on IKKα activity (Oeckinghaus and Ghosh 2009; Oeckinghaus et al. 2011; Sun 2011; DiDonato et al. 2012; Hayden and Ghosh 2012), which induces the proteolytic processing of the p52 precursor, p100, and nuclear entry of p52/RelB heterodimers and is largely involved in developmental roles, including survival of premature B cells and development of secondary lymphoid organs (Sun 2011). These functions have been demonstrated in $RelB^{-/-}$ and $IKK\alpha^{-/-}$ knockout mice, which both suffer from defective secondary lymphoid organ development (Weih et al. 1995; Weih et al. 1997; Yamada et al. 2000; Senftleben et al. 2001; Weih et al. 2001; DiDonato et al. 2012). Although both pathways of NF-κB activation are capable of regulating cell survival and cell death, it is the classical NF-κB pathway that is primarily responsible for inhibition of PCD under most circumstances (e.g., in the context of inflammation) (Oeckinghaus and Ghosh 2009; Oeckinghaus et al. 2011; Hayden and Ghosh 2012). A third pathway leading to NF-κB activation is triggered by genotoxic stresses that result in DNA damage through the atypical NF-κB activation pathway, which does not fall in the abovementioned canonical and noncanonical pathways (Janssens and Tschopp 2006). An IKK-independent pathway for UV-induced NF-κB activation has also been characterized that involves casein kinase 2 (CK2) (Teitz et al. 1990; Bender et al. 1998; Kato et al. 2003). UV exposure activates CK2 via p38 MAPK, which acts as an allosteric CK2 regulator. Activated CK2 then directly phosphorylates IκBα, triggering its degradation by the proteasome, resulting in NF-κB activation (Kato et al. 2003).

12.3 The Prosurvival Role of NF-κB in Animal Physiology

NF-κB transcription factors are central players in a system that allows cells to adapt and respond to environmental changes, a process pivotal for survival of organisms. The prosurvival function of NF-κB has been implicated in a wide range of biological processes, and the role of NF-κB in PCD is of critical importance in the development and homeostasis of the immune system (Li and Verma 2002; Bonizzi and Karin 2004). NF-κB guides the development of T and B lymphocytes, ensuring their survival at critical stages in their developmental program, thereby allowing progression to functional maturity. In the B cell lineage, NF-κB is constitutively activated by the triggering of the members of the TNFα receptor (TNF-R) family, transmembrane activator and calcium-modulating cyclophilin ligand

interactor, B cell maturation antigen, and B cell activating factor receptor (Mackay and Browning 2002), and this constitutive NF-κB activity is necessary for differentiation and maintenance of mature IgM^{low}/IgD^{high} B lymphocytes (Bonizzi and Karin 2004; Hayden and Ghosh 2004; Siebenlist et al. 2005). NF-κB also plays an important protective role in the development of thymocytes, and is required in the periphery for the productive responses of mature B and T lymphocytes following exposure to antigen and costimulatory molecules such as CD40 ligand and B7-1, respectively (Li and Verma 2002; Bonizzi and Karin 2004; Weil and Israel 2004; Siebenlist et al. 2005). The classical and alternative pathways of NF-κB activation antagonize PCD signaling triggered downstream of numerous receptors, such as Toll-like receptors and death receptors, including Fas and TNF-R1 (Bonizzi and Karin 2004; Hayden and Ghosh 2004), found on the surface of cells of the immune system. Various knockout mice models for NF-κB signaling components display defective lymphopoiesis and function due to an inability to protect developing and mature B and T lymphocytes from apoptosis induced by high levels of TNFα, owing to a lack of sufficient NF-κB activity to overcome cell death pathways (Li and Verma 2002; Bonizzi and Karin 2004).

The NF-κB-mediated control of PCD is also crucial to the physiology of organs outside the immune system. The first direct evidence that NF-κB inhibited PCD was provided by the observation that ablation of RelA in mice causes embryonic lethality owing to massive apoptosis in the liver (Beg et al. 1995). The role of NF-κB in embryonic hepatic cell survival was further underscored by similar phenotypes of mice that lack IKKβ, IKKγ, or RelA (Li et al. 1999; Rudolph et al. 2000; Kucharczak et al. 2003). Notably, both liver damage and embryonic lethality in mutant mice were reversed by the compound mutation of TNF-R1, confirming that liver apoptosis depends on signaling via this receptor (Doi et al. 1999; Alcamo et al. 2001). The hepatoprotective activity of NF-κB was also confirmed in adult animals (Chaisson et al. 2002; Maeda et al. 2003; Papa et al. 2006), with the demonstration that NF-κB activity is essential for antagonism of TNF-R-mediated damage. The protective action of NF-κB against PCD has been implicated in several other processes, including epidermal homeostasis, hair follicle development, and the development and function of the central nervous system (Mattson and Camandola 2001; Bell et al. 2003; Kucharczak et al. 2003).

12.4 NF-κB-Mediated Inhibition of PCD in Human Disease

As a consequence of the extensive range of stimuli inducing NF-κB activation, and of the multitude of target genes NF-κB induces, deregulation of NF-κB activity has been implicated in numerous diseases. NF-κB controls cell survival through the antagonism of PCD, and this suppressive action on PCD is crucial in carcinogenesis. Genes encoding NF-κB/IκB family members are frequently amplified, rearranged, or mutated in certain human cancers, and most cellular and viral oncogene products, including Bcr-Abl, Her-2/Neu, and oncogenic H-Ras and

K-Ras, are capable of eliciting NF-κB activation (Karin et al. 2002; Courtois and Gilmore 2006; Staudt 2010; Ben-Neriah and Karin 2011; DiDonato et al. 2012). Additionally, mutations that result in NF-κB activation in malignant cells occur in genes coding for signaling proteins that feed into the IKK–NF-κB axis or cause NF-κB to be activated by exposure to proinflammatory cytokines in the tumor microenvironment (Karin et al. 2002; Courtois and Gilmore 2006; Staudt 2010; Ben-Neriah and Karin 2011; DiDonato et al. 2012). Direct evidence from various in vivo and in vitro models now indicates that the NF-κB-mediated suppression of PCD is crucially involved in various aspects of cancer biology, including malignant transformation, tumor progression, and resistance to cancer therapy (Karin et al. 2002; Courtois and Gilmore 2006; Baud and Karin 2009; Li and Sethi 2010; Staudt 2010; Ben-Neriah and Karin 2011; DiDonato et al. 2012). Indeed, constitutive NF-κB activity is required for the survival of several cancerous cells, such as those in diffuse B cell lymphoma (DLBCL), acute lymphoblastic leukemia, multiple myeloma, Hodgkin's lymphoma, breast cancer, and many other hematological and solid tumors (Davis et al. 2001; Courtois and Gilmore 2006; Annunziata et al. 2007; Gilmore 2007; Jost and Ruland 2007; Compagno et al. 2009; DiDonato et al. 2012).

Mutations that give rise to constitutive NF-κB are best characterized in B cell malignancies. NF-κB is constitutively activated in activated B cell like (ABC)-DLBCL but not in germinal center B cell like DLBCL and results in increased B cell proliferation and survival even after the initial antigenic stimulus has ceased (Davis et al. 2001). Mutations in the *CARD11* gene were discovered in a subset of ABC-DLBCL, and result in a protein product that is a constitutive activator of the IKK/NF-κB pathway (Lenz et al. 2008; Compagno et al. 2009). In other ABC-DLBCLs, mutations that modify the Toll-like receptor adaptor protein, MyD88, have been identified which also result in constitutive NF-κB activity (Ngo et al. 2011). Furthermore, mutations involving the NF-κB pathway are present in at least 20 % of multiple myeloma tumors (Annunziata et al. 2007; Gilmore 2007). Although activated NFκB is a common feature of this plasma cell malignancy, no mutations in NF-κB- or IκB-encoding genes were discovered in this disease. Rather, a number of mutations in genes encoding upstream signaling molecules that lead to the stabilization and accumulation of NF-κB-inducing kinase, a member of the MAPK kinase kinase (MAP3K) family causing activation of both the classical and the alternative NFκB signaling pathway, were identified (Annunziata et al. 2007; Gardam and Beyaert 2011).

Recent studies have shed new light on the basis for the pivotal role of NF-κB in carcinogenesis. One common epigenetic event in cancer is inflammation, and indeed, chronic infections and inflammation account for 15–20 % of all cancer deaths in humans (Coussens and Werb 2002; He and Karin 2011; DiDonato et al. 2012). Hepatocellular carcinoma (HCC), for instance, often results from cirrhosis or chronic infection with hepatitis B virus or hepatitis C virus (Alison et al. 2011). Notably, it was shown that a crucial link between inflammation and carcinogenesis depends on NF-κB and that this is mediated by at least two mechanisms. Firstly, within precancerous/cancerous cells, NF-κB upregulates prosurvival

genes that enable these cells to evade PCD and propagate their malignant phenotype. Secondly, in nonparenchymal cells, NF-κB induces proinflammatory cytokines (e.g., TNFα, IL-6) that stimulate cancer cell growth (Ben-Neriah and Karin 2011; He and Karin 2011; DiDonato et al. 2012). Furthermore, NF-κB has recently been discovered to play an additional role in this link, via the regulation of the differentiation/function of so-called tumor-associated macrophages, which infiltrate tumors and promote cancer growth, angiogenesis, and metastasis formation (Hagemann et al. 2009; Mancino and Lawrence 2010). NF-κB activation was demonstrated to maintain the alternative immunosuppressive phenotype (M2 phenotype) of tumor-associated macrophages and negatively regulate macrophage tumoricidal activity (M1 phenotype) (Biswas et al. 2008; Hagemann et al. 2008).

Various experimental models have highlighted the importance of NF-κB in the link between cancer and inflammation. In a mouse model of colitis-associated cancer (CAC), cell-specific ablation of IKKβ within intestinal epithelial cells (i.e., enterocytes) or myeloid cells demonstrated that IKKβ-driven NF-κB activity contributes to the development of CAC through two distinct cell-type-specific mechanisms (Greten et al. 2004). Selective inactivation of the *Ikbkb* gene within enterocytes resulted in an 80 % reduction in CAC tumor multiplicity (Greten et al. 2004). However, tumor size was not affected in this animal model, indicating that IKKβ-mediated NF-κB activity in enterocytes contributes to tumor initiation and/or early tumor promotion, rather than to tumor progression, most likely through the activation of antiapoptotic genes and consequent suppression of apoptotic elimination of preneoplastic cells (Greten et al. 2004). Conversely, deletion of IKKβ in myeloid cells, but not in enterocytes, resulted in only a 50 % reduction in tumor multiplicity, but tumor volume was significantly reduced. Hence, it was concluded that in myeloid cells NF-κB promotes the production of cytokines that act as growth factors for premalignant enterocytes. One of these growth factors was subsequently identified as being IL-6, which is encoded by an NF-κB target gene. Indeed, the inhibition of IL-6 signaling resembled IKKβ ablation in myeloid cells, as tumor growth was inhibited, with little effect on tumor multiplicity (Greten et al. 2004; Grivennikov et al. 2009). Another mouse model of inflammation-driven carcinogenesis highlighting the role of NF-κB in the tumor microenvironment is the multidrug resistance 2 (MDR2)-knockout mouse, in which the absence of the MDR2 phospholipid transporter leads to the accumulation of bile acids and phospholipids within hepatocytes, resulting in low-grade hepatic inflammation which ultimately gives rise to HCC (Mauad et al. 1994). Inhibition of hepatocyte NF-κB through the expression of a nondegradable variant of IκBα blocked the development of HCC and enhanced apoptosis in premalignant hepatocytes (Pikarsky et al. 2004). Although the initial stimulus leading to NF-κB activation in *mdr2*$^{-/-}$ mice was not defined, it appears to be associated with a chronic inflammatory response to free bile acids that is propagated via paracrine TNF production. This is because the administration of a neutralizing anti-TNFα antibody was shown to inhibit NF-κB activation in hepatocytes and to diminish expression of NF-κB-dependent antiapoptotic genes (Pikarsky et al. 2004).

The role of NF-κB in cancer is not always protumorigenic. In a study by Maeda et al. (2005) of diethylnitrosamine-induced HCC requires NF-κB activation in myeloid cells. The specific ablation of IKKβ in Kupffer cells (resident liver macrophages) resulted in a downregulation of inflammatory cytokines such as IL-6 and TNFα and hepatomitogens needed for the growth and survival of tumor cells, with concomitant reduction in tumor load (Maeda et al. 2005). By contrast, hepatocyte-specific ablation of IKKβ sensitized diethylnitrosamine-treated premalignant hepatocytes to increased cell death. However, owing to the regenerative capacity of the liver, increased cell death led to compensatory proliferation of initiated cells and an enhancement of HCC (Maeda et al. 2005). Hence, in this model of HCC, NF-κB inactivation in the liver resulted in an increase of HCC incidence, owing to the activation of Kupffer cells and concomitant release of proinflammatory mediators following the detection of necrotic debris, thereby supporting hepatocyte regrowth and the transmission of oncogenic mutations (Maeda et al. 2005). This compensatory proliferation is required for fixation of oncogenic mutations that would otherwise be lost in the absence of cell proliferation. Subsequent work by He et al. (2010), using an experimental system that involved the transplantation of diethylnitrosamine-initiated hepatocytes into MUP-uPA mouse liver, allowed the examination of factors and mechanisms that affect the progression of initiated, preneoplastic hepatocytes into full-blown HCC, demonstrating that hepatocyte IKKβ-driven NF-κB suppresses malignant progression. IKKβ-driven NF-κB was shown to reduce late tumor promotion and progression of initiated hepatoma cells in this model, by preventing the accumulation of ROS that resulted in activation of the oncogenic transcription factor, STAT3, critical for HCC development (He et al. 2010). Other mouse models indicating a tumor suppressor function for NF-κB include studies on the role of NF-κB in the skin, whereby disrupting NF-κB activity in keratinocytes was shown to induce squamous cell carcinoma. RelA null and IKKβ null mice as well as transgenic mice constitutively expressing IκB-SR in the epidermis develop keratinocyte hyperplasia, followed by development of squamous cell carcinoma. Development of squamous cell carcinoma in these mouse models is dependent on TNF-R1 signaling, as TNF-R1 blockade resulted in the restoration of a normal epidermis (van Hogerlinden et al. 1999; Lind et al. 2004; Pikarsky and Ben-Neriah 2006).

In addition to the roles of NF-κB in tumorigenesis, the protective activity of NF-κB can hamper tumor cell killing inflicted by radiation or chemotherapeutic drugs and in doing so promote cancer resistance to cancer treatments. NF-κB regulates several genes, including those encoding Bcl-2, Bcl-xL, X-chromosome-linked inhibitor of apoptosis (XIAP), survivin and Akt, whose expression has been reported to mediate chemoresistance and radioresistance in various tumor cells (Baud and Karin 2009; Li and Sethi 2010). Ionizing radiation has been reported to activate NF-κB in both in vitro and in vivo models, whereas several chemotherapeutic agents including paclitaxel, vinblastine, vincristine, doxorubicin, daunomycin, 5-fluorouracil, cisplatin, tamoxifen, and bortezomib have been reported to induce NF-κB activation in different cell types (Baud and Karin 2009; Li and Sethi 2010). Accordingly, the development of several classes of NF-κB inhibitors

for use in conjunction with chemotherapy and radiotherapy could be of enormous benefit in terms of blocking the various steps leading to NF-κB activation, and thereby sensitize tumor cells to the beneficial effects of chemotherapeutic drugs and radiation.

In addition to cancer, the NF-κB protective activity is critically involved in the pathogenesis of chronic inflammatory diseases such as rheumatoid arthritis, psoriasis, and inflammatory bowel disease whereby inflammation is sustained by a positive-feedback loop between TNFα and NF-κB. Compelling genetic, biochemical, and clinical evidence now demonstrates that inflammation is a key etiopathogenetic factor also in the development of metabolic diseases as well as of the complications that emerge from these diseases, particularly in the context of obesity, insulin resistance, type 2 diabetes, and atherosclerosis (Hotamisligil 2006; Baker et al. 2011). Consistent with its roles in immunity and inflammation, several studies have highlighted the key role that the IKK/NF-κB signaling pathway plays in the development of inflammation-associated metabolic diseases. Consequently, the NF-κB-mediated inhibition of PCD could play an important contributing role in the onset of metabolic disorders (Hotamisligil 2006; Baker et al. 2011).

Certain inherited conditions are also characterized by constitutive activation of NF-κB, including familial expansile osteolysis and familial Paget disease of bone, both caused by hypermorphic mutations of receptor activator of NF-κB and an exaggerated survival and function of osteoclasts (Orange et al. 2005). Conversely, an impairment in cell survival is a key pathogenetic element in incontinentia pigmenti—a rare X-linked disorder usually caused by rearrangements of the IKKγ/NEMO gene—and other genetic illnesses characterized by various defects in the NF-κB activation pathway, including primary immunodeficiencies and anhidrotic ectodermal dysplasia syndromes (Smahi et al. 2002; Orange et al. 2005).

12.5 TNF-R1-Induced Pathways of PCD

The prosurvival action of NF-κB was first discovered and is best understood in the context of the ligation and activation of the prototypical member of the "death receptors," TNF-R1, by TNFα (Wang and Lin 2008; Wajant and Scheurich 2011; Cabal-Hierro and Lazo 2012). TNFα is a pleiotropic cytokine that plays an important role in inflammation, immunity, cell growth, differentiation, and the induction of PCD (Wang and Lin 2008). There are two receptors for TNFα, namely, TNF-R1 and type 2 TNFα receptor (TNF-R2). TNF-R1 is ubiquitously expressed, whereas TNF-R2 is mainly expressed in immune cells (Wang and Lin 2008; Cabal-Hierro and Lazo 2012). Although both receptors bind to TNFα, the main receptor mediating the cellular effects of TNFα in most cell types is TNF-R1. TNFα signaling through its receptors can trigger cell death (Wang and Lin 2008; Cabal-Hierro and Lazo 2012). Normally, however, despite this well-documented ability to induce cell death, stimulation of TNF-R1 by TNFα does not result in cell death unless NF-κB activation or new RNA/protein synthesis is blocked (Kucharczak et al.

2003). Hence, depending on the cell type, cell activation state, and microenvironment factors, TNFα may result in cell survival, apoptosis, or necroptosis. This functional trichotomy of TNF-R1 reflects the existence of an intricate network of signals that operate downstream of TNF-R1 and which can "switch" between different patterns of response. Indeed, the biological outcome from the ligation of TNF-R1 depends on the sequential assembly and activation of multiproteic complexes (Vandenabeele et al. 2010; Wajant and Scheurich 2011; Cabal-Hierro and Lazo 2012) (see Fig. 12.1).

TNF-Rs lack intrinsic enzymatic activity, thus signaling by TNF-R1 and TNF-R2 is achieved by the recruitment of adaptor proteins that bind to their cytosolic region (Vandenabeele et al. 2010; Wajant and Scheurich 2011; Cabal-Hierro and Lazo 2012). On binding of a TNFα homotrimer to trimerized TNF-R1, the intracellular portion of TNF-R1 recruits the adaptor protein TNF-R-associated death domain (TRADD) through homotypic interaction between their death domains (a conserved protein–protein interaction motif of 80 amino acids contained in all death receptors) (Vandenabeele et al. 2010; Wajant and Scheurich 2011; Cabal-Hierro and Lazo 2012). TRADD serves as a platform to recruit downstream adaptor proteins and leads to the formation of a membrane-proximal supramolecular structure, that includes receptor-interacting protein (RIP) 1, TNF-R-associated factor (TRAF) 2, TRAF5, and cellular inhibitors of apoptosis (cIAPs) 1 and 2, termed complex I (Vandenabeele et al. 2010; Wajant and Scheurich 2011; Cabal-Hierro and Lazo 2012). The assembly of this complex triggers cellular pathways that lead to the activation of NF-κB, as well as the JNK cascade (Vandenabeele et al. 2010; Wajant and Scheurich 2011; Cabal-Hierro and Lazo 2012). Once this core complex is assembled, several ubiquitination events occur and promote the full assembly and function of complex I. Briefly, the term "ubiquitination" refers to the covalent attachment of the small protein ubiquitin to target proteins. Ubiquitination is a posttranslational modification that serves as a recognition signal and can regulate, activate, or inactivate proteins within signal transduction cascades (Emmerich et al. 2011; Schmukle and Walczak 2012). The ubiquitination reaction involves the action of a ubiquitin-activating enzyme (E1) and a ubiquitin-conjugating enzyme (E2), which in the presence of a ubiquitin ligase (E3) mediate the attachment of ubiquitin to lysine residues or the N terminus of a target protein (Emmerich et al. 2011; Schmukle and Walczak 2012). The E3 ubiquitin ligases, cIAPs, which were previously known as apoptosis inhibitors owing to their functions in caspase inactivation (Deveraux et al. 1998), are recruited to complex I by TRAF2, which stabilizes them by preventing their polyubiquitination (Csomos et al. 2009). cIAPs catalyze the attachment of Lys63-linked polyubiquitin chains to RIP1 (Bertrand et al. 2008), which allows the stabilization and recruitment of the IKK and transforming growth factor β activated kinase 1 (TAK1)–TAK1-binding protein (TAB) complexes (Hacker and Karin 2006). Recent studies have introduced further levels of complexity in terms of TNF-R signaling. The E3 ligase activity of cIAPs has also been demonstrated to be required for the efficient recruitment of an E3 ligase complex, known as the linear ubiquitin chain assembly complex (LUBAC) (Haas et al. 2009). LUBAC,

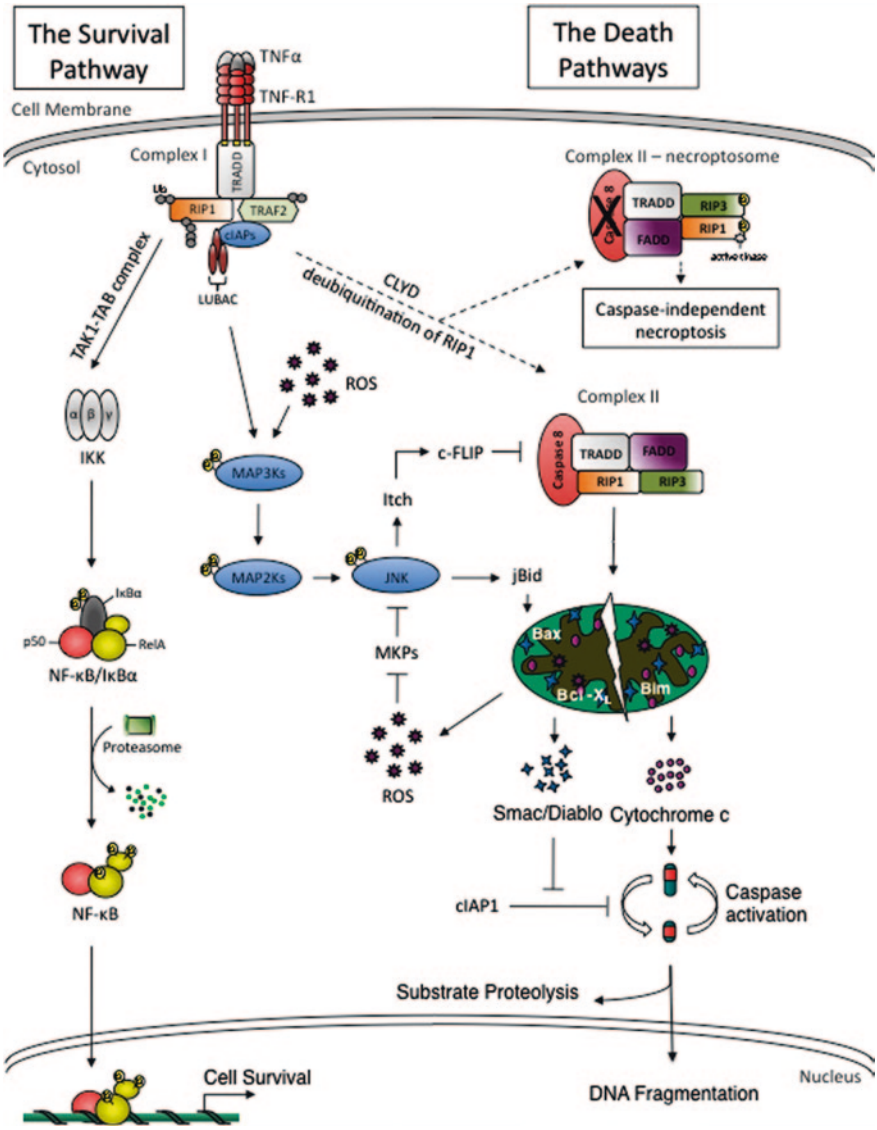

Fig. 12.1 Tumor necrosis factor α (*TNFα*) receptor 1 (*TNF-R1*)-induced pathways modulating cell death and survival. Ligation of TNF-R1 by TNFα promotes the intracellular assembly of complex I, which includes TNF-receptor-associated death domain (*TRADD*), receptor-interacting protein 1 (*RIP1*), TNF-receptor-associated factor 2 (*TRAF2*), and cellular inhibitors of apoptosis (*cIAPs*) 1 and 2. The cIAP-mediated Lys63 ubiquitination of RIP1 allows the recruitment of the inhibitor of nuclear factor κB (*IκB*) kinase (*IKK*) and transforming growth factor β activated kinase 1 (*TAK1*)–TAK1-binding protein (*TAB*) complexes. Complex I can also bind the linear ubiquitin chain assembly complex (*LUBAC*), which induces ubiquitin modifications resulting in the further stabilization of complex I, thereby allowing enhanced IKK activation. The activated IKK complex phosphorylates IκBα, with subsequent ubiquitination and degradation by the

proteasome, allowing the nuclear translocation and activation of NF-κB, which comprises the subunits p50 and RelA. NF-κB activates the transcription of an array of target genes that ultimately lead to cell survival. Complex I can also activate the c-Jun N-terminal kinase (*JNK*) signaling pathway through the recruitment and phosphorylation of mitogen-activated protein kinase kinase kinases (*MAP3Ks*) (TAK1 and apoptosis-signal-regulating kinase), which in turn activate mitogen-activated protein kinase kinases (*MAP2Ks*) (MKK4 and MKK7), leading to activation of JNK. JNK phosphorylates Itch, which targets cellular FLICE-inhibitory protein (*c-FLIP*) for ubiquitination and degradation, thereby promoting caspase 8 dependent apoptosis. JNK can also induce mitochondrial-dependent apoptosis through the activation of proapoptotic proteins jBid, Bim, and Bax. Deubiquitination of RIP1 by cylindromatosis (*CYLD*) results in the internalization of the TNF-R1 complex and modification of its binding partners, which leads to the cytosolic assembly of complex II. Complex II comprises TRADD, Fas-associated death domain (*FADD*), RIP1, receptor-interacting protein 3 (*RIP3*), and caspase 8. Caspase 8 cleaves and inactivates RIP1 and RIP3 kinases to preclude necroptosis, and initiates apoptosis by activating the classical caspase cascade. Under certain conditions in which caspase 8 activation is inhibited, RIP1 and RIP3 are phosphorylated and a unique signaling complex termed the necroptosome is formed, which leads to cell death through a caspase-independent process known as necroptosis. *MKP* mitogen-activated protein kinase phosphatase, *ROS* reactive oxygen species, *Smac* second mitochondria-derived activator of caspases

consisting of the HOIL-1, HOIP, and Sharpin proteins, generates linear ubiquitin chains through the α-amino group of the N-terminal methionine residue of the ubiquitin molecule (Emmerich et al. 2011; Schmukle and Walczak 2012). LUBAC binds linear ubiquitin chains to RIP1 and NEMO (IKKγ), leading to an overall stabilization and extension of the half-life of complex I, thereby allowing enhanced IKK activation (Gerlach et al. 2011). The activated IKK complex leads to phosphorylation of IκBα, primarily mediated by IKKβ, with subsequent ubiquitination and degradation by the proteasome, allowing the liberated NF-κB to translocate to the nucleus and activate transcription of an array of target genes that, ultimately, promote cell survival. Indeed, reduction of the various components of LUBAC (HOIL-1, HOIP, and Sharpin) through genetic ablation demonstrates that absence of LUBAC results in reduced NF-κB activation, and increased TNFα-induced cell death (Emmerich et al. 2011; Gerlach et al. 2011; Schmukle and Walczak 2012).

In addition to NF-κB, TNF-R1 signaling leads to the activation of the JNK MAPK signaling cascade (see Fig. 12.1). RIP1 is also required for efficient JNK activation (Devin et al. 2003) and a number of MAP3Ks have been implicated in the activation of JNK downstream of TNF-R1 on the basis of the induction of their activity by TNFα and the phenotypes of various knockout model systems, including MEKK1, apoptosis-signal-regulating kinase 1 (ASK1), and TAK1 (Sadoshima et al. 2002; Tang et al. 2008; Soberanes et al. 2009). MEKK1 and TAK1 have both been proposed to be essential for TNFα-induced JNK activation, on the basis of signaling in deficient embryonic fibroblasts (Xia et al. 2000; Shim et al. 2005). Complex I recruits and phosphorylates MAP3Ks, which in turn activate MKK4 and MKK7, leading to activation of JNK (Wang and Lin 2008; Wajant and Scheurich 2011; Cabal-Hierro and Lazo 2012). Despite the initial controversy in

the role of JNK activation in TNF-induced cell death, it is now clear that in most cases sustained JNK activation is proapoptotic.

The internalization of the TNF-R1 complex and modification of its conformation ensure the formation of a cytosolic, death-inducing signaling complex, better known as complex II, which leads to the promotion of either apoptosis or necroptosis (Vandenabeele et al. 2010). The key event in the apoptotic signaling pathway of cell death is the activation of caspase proteases, which are cysteine proteases that cleave specific cellular substrates, leading to cell death (Wen et al. 2012). Normally these events result in cytoplasmic shrinkage, chromatin condensation and nuclear fragmentation, plasma membrane blebbing, and the formation of membrane-bound bodies containing the cellular structures and organelles (Wen et al. 2012). Complex II formation occurs on deubiquitination of RIP1 by the enzyme cylindromatosis, leading to the dissociation of the TRADD–RIP1–TRAF2 complex from TNF-R1 (Hitomi et al. 2008). Caspase activation during TNFα-mediated apoptosis is achieved through the recruitment of Fas-associated death domain (FADD) and RIP3 (kinase cognate to RIP1) to this secondary complex (Micheau and Tschopp 2003; Wen et al. 2012). FADD recruits and causes autocatalytic activation of the initiator caspases 8 and 10 (Micheau and Tschopp 2003; Wen et al. 2012). In complex II, caspases 8 and 10 cleave and inactivate RIP1 and RIP3 kinases to preclude necroptosis, and initiate the proapoptotic caspase activation cascade (Feng et al. 2007; Declercq et al. 2009; He et al. 2009). In certain cells, activation of this extrinsic death receptor pathway is insufficient to induce cell death, and requires a mitochondrial amplification loop (the intrinsic apoptotic pathway). Mitochondrial function is an important checkpoint in PCD and mitochondrial integrity is necessary for maintenance of cellular homeostasis. Loss of mitochondrial integrity and the release of mitochondrial apoptogenic proteins, such as cytochrome *c* and second mitochondria-derived activator of caspases (Smac), as a result of increased mitochondrial outer membrane permeabilization, is a trigger for apoptosis (Micheau and Tschopp 2003; Declercq et al. 2009; Wen et al. 2012). Proapoptotic members of the Bcl-2 family of proteins, such as Bid (in its truncated tBid form following proteolytic cleavage by caspase 8), Bax, and Bak, positively regulate cytochrome *c* release in response to death stimuli by acting directly on the outer mitochondrial membrane (Micheau and Tschopp 2003; Declercq et al. 2009; Wen et al. 2012). The subsequent disruption of mitochondrial membrane potential results in the release of cytochrome *c* and Smac/DIABLO from the mitochondrial intermembrane space into the cytoplasm (Micheau and Tschopp 2003; Wen et al. 2012). Newly released cytochrome *c*, along with dATP, binds the adaptor protein apoptotic protease-activating factor 1, which leads to the formation of the so-called apoptosome complex that recruits and activates the initiator caspase 9. Activated caspase 9 in turn activates effector caspases 3 and 7, leading to the cleavage of protein substrates and apoptotic cell death (Micheau and Tschopp 2003; Wen et al. 2012).

In addition to apoptosis, TNFα is capable of activating necroptosis, a form of caspase-independent cell death whose molecular regulation is poorly understood

(Declercq et al. 2009; Vandenabeele et al. 2010). Cellular swelling, organelle dysfunction, extensive mitochondrial damage, and extensive plasma membrane rupture characterize necroptosis (Declercq et al. 2009; Vandenabeele et al. 2010). Failure of energy metabolism and massive generation of ROS are each thought to promote necroptosis (Declercq et al. 2009; Vandenabeele et al. 2010). Indeed, ROS can play a role in both apoptosis and necroptosis, whereby moderate oxidative stress generally enhances the execution of apoptosis , whereas high exposure to ROS results in necroptosis (Saito et al. 2006). An important physiological consequence of the decision whether to undergo apoptosis or necroptosis is that whereas necrotic cells release factors such as chromatin-associated HMG1 and heat shock protein 70, which serve as potent stimuli for inflammation (El Mezayen et al. 2007), apoptotic cells suppress immunity and are rapidly engulfed by phagocytes with little inflammation, indicating a potential physiological role for necroptosis in inflammation and antiviral host defense (Cho et al. 2009). Recent studies have revealed that following caspase 8 inhibition (by pharmacological inhibition or under certain biological circumstances such as cellular stress), complex II is unable to trigger apoptosis (Vercammen et al. 1998; Holler et al. 2000). Under these conditions, the activation of TNF-R1 leads to the formation of a unique pronecrotic complex II (necroptosome), which leads to necroptosis in a cell-type-specific manner (see Fig. 12.1). Although TRADD and FADD seem to be present in this multiprotein complex (Holler et al. 2000), various studies have shown that mutual phosphorylation of RIP1 and RIP3 is the crucial step in the stable assembly of this complex and in the induction of necroptosis (Cho et al. 2009; He et al. 2009). Unlike its role in the activation of NF-κB and JNK, the pronecrotic role of RIP1 requires its kinase activity (Declercq et al. 2009). Indeed, Degterev et al. (2008) identified the small molecules necrostatin 1 and necrostatin 2 as specific inhibitors of RIP1 kinase activity (Vercammen et al. 1998). These molecules inhibited necroptosis, but the RIP1-mediated activation of NF-κB and JNK was unaffected (Vercammen et al. 1998). The distinct molecular mechanism by which the RIP1/RIP3-assembled necroptosome contributes to the execution of TNF-R1-initiated necroptosis is presently unclear. However, most studies are focusing on its potential link to mitochondrial disruption and exacerbation of ROS release. The first link between mitochondrial energy metabolism and the execution of necroptosis was established in the early 1990s, when Schulzeosthoff et al. (1992) demonstrated that ROS production by mitochondrial respiratory complex I is essential for the necrotic response of L929 cells to TNFα (Schulzeosthoff et al. 1992). Although ROS production is not essential in all cases for TNF-induced necroptosis, the kinase activity of RIP3 may potentially link TNF-R1 signaling, mitochondrial energy metabolism, and ROS overproduction. Although the downstream effects of the RIP3 kinase activity are uncertain, RIP3 has been demonstrated to physically interact with and activate several enzymes within metabolic pathways, including glycogen phosphorylase, glutamate–ammonia ligase, and glutamate dehydrogenase, leading to increased energy-metabolism-associated ROS production. Indeed, RNA interference mediated knockdown of

any of these enzymes attenuates TNFα-mediated ROS production and necroptosis (Zhang et al. 2009).

12.6 The JNK-MAPK Cascade

The JNK pathway, also formerly known as the stress-activated protein kinase pathway, is one of the major MAPK cascades mediating the intracellular transduction of signals. Like the other two main MAPK cascades—the p38 and the extracellular-signal-regulated kinase cascades—the JNK cascade transduces signals triggered by diverse stimuli through the sequential phosphorylation of hierarchically arranged kinase modules in order to elicit an appropriate cellular response (Karin and Gallagher 2005; Dhanasekaran and Reddy 2008; Seki et al. 2012). The JNK signaling pathway is activated through a three-tier kinase cascade that includes multiple MAP3Ks, two MAPK kinases (MAP2Ks) (MKK4 and MKK7), and three JNKs depending on the tissue, stimulus, and biological context (Karin and Gallagher 2005; Dhanasekaran and Reddy 2008; Seki et al. 2012). In mammals, three genetic loci encode the terminal enzymatic modules of this cascade—JNK1, JNK2, and JNK3—each of which has two to four isoforms that result from the alternative splicing of the corresponding precursor messenger RNAs (Karin and Gallagher 2005; Dhanasekaran and Reddy 2008; Seki et al. 2012). JNK1 and JNK2 are ubiquitously expressed, whereas JNK3 is expressed predominantly in the brain and to a lesser extent in the heart and testis (Karin and Gallagher 2005; Dhanasekaran and Reddy 2008; Seki et al. 2012). JNKs are primarily activated on exposure to proinflammatory cytokines such as TNFα and IL-1β or stress stimuli such as UV radiation and genotoxic, osmotic, oxidative, and hypoxic stress (Karin and Gallagher 2005; Dhanasekaran and Reddy 2008; Seki et al. 2012). Induction of the JNK cascade by these stimuli has been linked to different biological responses, including growth and differentiation. However, a large body of evidence exists that suggests that the activation of JNK is in most cases a trigger for cell death and inflammation.

Evidence for the involvement of JNK in PCD signaling originated from studies using mouse embryonic fibroblasts (MEFs) derived from *jnk1*$^{-/-}$ and *jnk2*$^{-/-}$ knockout mice. MEFs lacking both JNK1 and JNK2 demonstrated resistance to apoptosis in response to UV irradiation (Tournier et al. 2000). This defect in apoptosis was correlated with a lack of cytochrome *c* release from mitochondria and subsequent caspase activation. Moreover, thymocytes and peripheral T cells from both *jnk1*$^{-/-}$ and *jnk2*$^{-/-}$ mice are seemingly protected against anti-CD3-induced death and activation-induced cell death, respectively (Tournier et al. 2000; Karin and Gallagher 2005). Consistent with this proapoptotic role of JNK is the observation that T cells deprived of either JNK1 or the JNK MKK7/JNKK2 mount an exaggerated proliferative reaction to antigen stimulation (Dong et al. 1998; Tournier et al. 2000; Sasaki et al. 2001; Karin and Gallagher 2005). The role of JNK3 in apoptosis was also established using *jnk3*$^{-/-}$ mice. These mice were

observed to be resistant to excitotoxic agent (glutamate, kainite) induced apoptosis of their hippocampal neurons compared with wild-type control mice (Yang et al. 1997).

Despite this large body of work indicating a proapoptotic role for JNK, some initial studies had suggested that JNK activation by TNFα either enhanced or did not affect cell survival (Liu et al. 1996; Natoli et al. 1997). This issue has now been clarified by the analyses of JNK- and NF-κB-deficient models. The use of these models has brought about a general agreement that JNK signaling plays an obligatory role in TNF-R-inflicted killing in many systems, and that the targeting of JNK signaling is an important means by which NF-κB suppresses PCD (Bubici et al. 2004; Papa et al. 2004, 2006). Normally, cells survive TNFα treatment because TNFα triggers potent, but only transient activation of the JNK cascade, with basal levels rapidly reestablished generally within 30–60 min (De Smaele et al. 2001; Javelaud and Besancon 2001; Tang et al. 2001). Blocking NF-κB activity, however, by either the knockout deletion of RelA or IKKβ or ectopic expression of IκBαM, a degradation-resistant variant of IκBα, markedly impairs the normal shutdown of TNFα-induced JNK signaling, thereby unveiling an additional sustained phase of JNK signaling (De Smaele et al. 2001; Javelaud and Besancon 2001; Tang et al. 2001). Indeed, it is this prolonged phase of JNK induction by TNFα that has been implicated in the activation of PCD (De Smaele et al. 2001; Javelaud and Besancon 2001; Tang et al. 2001). Therefore, in the presence of NF-κB, JNK activity is elevated by TNFα only transiently, which explains why this transient activation occurs without significant cytotoxicity. In NF-κB-deficient cells, however, JNK induction by TNFα remains sustained even after antiapoptotic treatment with the caspase blocker Z-VAD_{fmk} (Javelaud and Besancon 2001; Papa et al. 2004). Hence, the inhibitory effects of NF-κB on the JNK pathway do not appear to be an indirect consequence of suppressing the activation of caspases, despite the fact that these proteases are potentially capable of activating MAP3Ks (Wen et al. 2012). This ability of NF-κB to control the sustained activation of JNK signaling is a pivotal protective mechanism against TNF-R-induced cytotoxicity.

Consistent with this concept, suppression of JNK signaling by either pharmacological means or expression of dominant negative kinase mutants effectively rescues NF-κB-deficient cells from TNFα-induced cell death (De Smaele et al. 2001; Javelaud and Besancon 2001; Tang et al. 2001). Likewise, either compound deletion of JNK1 and JNK2 or the silencing of MKK7 expression virtually abrogates TNFα-induced death in *relA*$^{-/-}$, *ikkβ*$^{-/-}$, or IκBαM-expressing cells (Deng et al. 2003; Ventura et al. 2004; Kamata et al. 2005). Further evidence highlighting the biological relevance of this antagonist cross talk between the NF-κB and JNK pathways in promoting cell survival was demonstrated in vivo. Analyses of IKKβ/JNK1 double mutants showed that the absence of JNK1—one of the two major JNK isoforms expressed in the liver—delayed lethality in embryonic mice because of compromised NF-κB activation, suggesting that JNK signaling is an important mediator of apoptosis in the liver during embryogenesis and that the suppression of JNK signaling is a key mechanism by which NF-κB promotes survival during liver development (Chang et al. 2006). In a mouse model

of TNFα-mediated liver injury caused by the systemic administration of concavalin A (ConA), NF-κB induced antiapoptotic effects by attenuating JNK activation (Maeda et al. 2003). This was demonstrated by the observations that hepatocyte-specific deletion of IKKβ enhances the activation of JNK and that suppression of this response by the additional ablation of either JNK1 or JNK2 reduces ConA-induced liver injury (Maeda et al. 2003). Generation of double-mutant mice harboring specific inhibition of both IKKβ and JNK1 (IKKβ$^{\Delta hep}$/*jnk1*$^{-/-}$) provided additional evidence for cross talk between the NF-κB and JNK pathways in promoting cell survival. Indeed, JNK1 deletion reversed increased hepatocyte death and the susceptibility of mice to HCC development in the absence of IKKβ (Sakurai et al. 2006). Hence, the available evidence indicates that an abrogation of sustained activation of the JNK cascade is crucial for control of TNFα-induced PCD, and furthermore, that this abrogation is critically dependent on NF-κB.

12.7 How Does JNK Activation Execute Cell Death?

A mechanistic site of action for JNK in apoptosis appears to be in the mitochondria. The most conclusive evidence for this role comes from a study where *jnk1*$^{-/-}$ and *jnk2*$^{-/-}$ primary MEFs were found to be resistant to UV radiation owing to protection against cytochrome *c* release and mitochondrial depolarization (Tournier et al. 2000). NF-κB activation inhibits the ability of TNF-R1 to induce apoptosis by maintaining high levels of cellular FLICE-inhibitory protein (cFLIP), a specific inhibitor of caspase 8/10 activation (Chang et al. 2006). Chang et al. (2006) demonstrated a link between JNK activity and cFLIP, whereby TNFα was found to induce proteosomal degradation of the antiapoptotic protein cFLIP in wild-type but not JNK1-deficient cells (Chang et al. 2006). JNK1, however, does not phosphorylate cFLIP directly and instead leads to the phosphorylation-dependent activation of the ubiquitin ligase Itch. Itch specifically interacts with the long isoform of cFLIP, cFLIP$_L$, promoting its polyubiquitination and subsequent proteosomal degradation (Chang et al. 2006). Since cFLIP$_L$ impairs the recruitment of procaspase 8 to complex II, the JNK1-induced degradation of cFLIP$_L$ allows full activation of caspase 8, resulting in cell death (Chang et al. 2006). Evidence also indicates that prolonged JNK activation promotes the processing of the BH3-domain protein Bid to the proapoptotic protein jBid (akin to caspase 8 cleaved Bid, tBid) (Deng et al. 2003). This proteolytic fragment, jBid, specifically induces the release of mitochondrial Smac/DIABLO, an apoptotic protein that displaces cIAP1 from the TNF-R complex and consequently allows caspase 8 activation and initiation of apoptosis (Deng et al. 2003). JNK has also been observed to modulate the activities of other proapoptotic BH3-domain-containing members of the Bcl-2 family, such as Bim and Bmf (Lei and Davis 2003). Bim and Bmf are released from sequestering complexes following their phosphorylation by JNK, and once released, phosphorylated Bim and Bmf play a role in activating the mitochondrial proteins Bax and Bak, thereby stimulating the release of cytochrome *c* from the mitochondrial

inner membrane, leading to caspase-dependent apoptosis (Lei and Davis 2003; Kim et al. 2006). In support of a role for JNK in this Bax/Bak-activating pathway, constitutively active JNK induces apoptosis in wild-type cells but not in cells deficient in Bax or Bak (Lei et al. 2002). In terms of the nuclear signaling of JNK in the regulation of apoptosis, phosphorylation and nuclear translocation of JNK results in the transactivation of c-Jun. Phosphorylation of c-Jun leads to the formation of activator protein 1 (AP-1), a transcription factor complex composed of homodimers and/or heterodimers of Jun and Fos proteins (Karin and Gallagher 2005; Seki et al. 2012). Early in vitro studies indicated that increased AP-1 activity could lead to apoptosis in specific cell types. It has been noted that the JNK/AP-1 pathway is involved in the increased expression of proapoptotic genes such as Bim, Fas ligand, and TNFα (Karin and Gallagher 2005; Seki et al. 2012).

12.8 Downstream Effectors of NF-κB-Mediated Control of JNK Signaling

The primary mechanism through which NF-κB secures an effective shutdown of the JNK pathway following the triggering of TNF-Rs involves the activation of a select subset of NF-κB target genes. These NF-κB-regulated genes have been shown to suppress JNK signaling via different mechanisms, as discussed in the following sections(see also Fig. 12.2).

*12.8.1 Gadd45*β

Following the implementation of a screen for complementary DNAs capable of blocking TNFα-induced PCD in *relA*$^{-/-}$ fibroblasts, Gadd45β/MyD118 was identified as a bone fide inhibitor of the JNK pathway (De Smaele et al. 2001). Gadd45β is a member of the growth arrest and DNA damage inducible (Gadd) 45 family of factors, which also includes Gadd45α and Gadd45γ (Yang et al. 2009). Gadd45 genes encode small (18 kDa), evolutionarily conserved proteins that are highly homologous to each other (55–57 % overall identity at the amino acid level), are acidic, and are primarily, but not exclusively, localized within the cell nucleus (Yang et al. 2009). Gadd45 proteins are induced under a wide variety of stress conditions, including cell cycle arrest, DNA repair, cell survival or apoptosis, and modulation of immune response (Yang et al. 2009).

Gadd45β is induced rapidly by TNFα and other inflammatory stimuli through a transcriptional mechanism that is dependent on NF-κB (De Smaele et al. 2001). The expression of Gadd45β is dependent on the binding of RelA-containing NF-κB complexes to three κB elements within the promoter region of its gene (Jin et al. 2002), and this may explain in part why *relA*$^{-/-}$ cells have been shown to be highly sensitive to TNFα-induced cell death and apoptosis induced by other

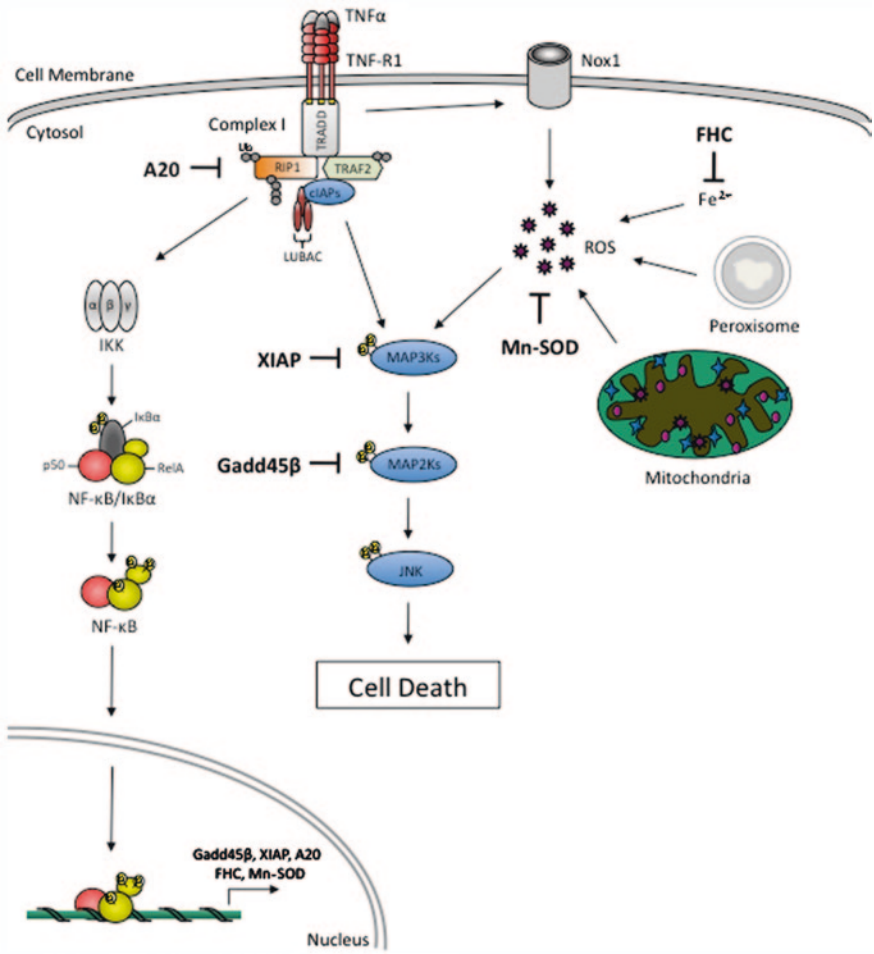

Fig. 12.2 The downstream effectors of NF-κB-mediated suppression of JNK and ROS activities and their proposed mechanisms of action.The main protective activity of NF-κB against TNF-R1-induced killing involves the activation of NF-κB-inducible target genes, which act to induce an effective shutdown of prolonged JNK cascade activation following the triggering of TNF receptors. One subset of NF-κB-inducible target genes, which includes Gadd45β, X-chromosome-linked inhibitor of apoptosis (*XIAP*), and A20, acts directly on the JNK cascade. Another subset of target genes, including ferritin heavy chain (*FHC*) and manganese superoxide dismutase (*Mn-SOD*), block prolonged JNK signaling by suppressing the accumulation of ROS derived from various sources, including the mitochondria, peroxisomes, NAPDH oxidases, and free iron

DNA-damaging agents (De Smaele et al. 2001; Jin et al. 2002). Indeed, the ectopic expression of Gadd45β rescued NF-κB null cells from TNFα-induced killing (De Smaele et al. 2001). Most importantly, the inactivation of endogenous Gadd45β by either expression of antisense messenger RNAs or targeted deletion of the

Gadd45β gene impairs cell survival and the downmodulation of JNK activity downstream of TNF-Rs, suggesting that in some tissues Gadd45β is required for cell survival through an NF-κB-mediated antagonism of JNK activation and PCD (De Smaele et al. 2001).

The Gadd45β-mediated inhibition of this JNK activation involves a direct blockade of the JNK MKK7/JNKK2, and this interaction between Gadd45β and MKK7 represents a crucial link between the NF-κB and JNK pathways (Papa et al. 2007). A model analysis of the Gadd45β/MKK7 complex predicts that Gadd45β binds to crucial residues in the catalytic pocket of MKK7, including the ATP-binding residue, Lys149, thereby obstructing its access to ATP (Papa et al. 2007; Tornatore et al. 2008). MKK7 is a selective activator of JNK, and its ablation in fibroblasts abolishes JNK induction by TNFα (Tournier et al. 2001). Thus, blocking this MAP2K is seemingly sufficient alone to account for the specific and effective inhibition of the JNK cascade by Gadd45β downstream of TNF-Rs. Although Gadd45β has also been shown to interact with other constituents of MAPK pathways, such as the MAP3Ks MEKK4/MTK1 and ASK1/MEKK5, it appears that these other interactions of Gadd45β are not involved in the control of JNK activation in the context of TNFα signaling (Papa et al. 2007). With regard to the in vivo role of Gadd45β in cytoprotection, Gadd45β was demonstrated to have an essential role in the protection against JNK-induced hepatocyte death in a mouse model of liver regeneration after partial hepatectomy (Papa et al. 2008). Gadd45β is consistently upregulated during the priming phase of liver regeneration following partial hepatectomy, a response that is coordinated by TNF-R1 through NF-κB/JNK cross talk (Papa et al. 2008). It was reported that whereas wild-type mice generally recover after partial hepatectomy, the survival rate of *gadd45β*$^{-/-}$ mice is dramatically reduced (Papa et al. 2008). These mice exhibited impaired hepatocyte proliferation and increased PCD during liver regeneration (Papa et al. 2008). Significantly, JNK and MKK7 activities were increased and sustained in the livers of *gadd45β*$^{-/-}$ mice compared with control mice after partial hepatectomy (Papa et al. 2008). Indeed, *JNK2* double-mutant mice harboring the compound deletion of *gadd45β* (*gadd45β*$^{-/-}$/*jnk2*$^{-/-}$) exhibited reduced hepatocyte death and increased proliferation, thereby corroborating the role of Gadd45β in promoting cell survival through the modulation of JNK-mediated PCD (Papa et al. 2008). Svensson et al. (2009) recently demonstrated that Gadd45β deficiency contributes to enhanced MKK7 and JNK responses in a mouse model of rheumatoid arthritis, leading to increased expression of matrix metalloproteinases in the rheumatoid arthritis synovial tissue, and an exacerbation of joint destruction (Svensson et al. 2009).

This body of work provides strong evidence that the protective activity of Gadd45β against TNFα-induced PCD involves suppression of the JNK cascade by means of MKK7 inactivation. Further evidence indicates that this Gadd45β-afforded suppression of JNK is crucial to the prosurvival activity of NF-κB. Gadd45β expression in MEF cells and 3DO T cells was elevated rapidly by TNFα through an NF-κB-dependent mechanism, which is essential for the antagonism of TNFα killing (Gupta et al. 2006; Larsen et al. 2006). This antiapoptotic role of

Gadd45β was further supported by work from other groups which demonstrated that overexpression of Gadd45β suppresses IL-1β induced apoptosis in insulin-producing cells, and that Gadd45β alleviated UV-induced apoptosis in hematopoietic cells by suppressing the JNK cascade (Gupta et al. 2006; Larsen et al. 2006). However, this antiapoptotic mechanism is subject to challenge by the findings of Engelmann et al. (2008), who demonstrated that high Gadd45β protein levels do not protect tumor cells against UV-radiation- or γ-radiation-induced cell apoptosis, but rather confer a strong and specific survival advantage to serum withdrawal (Engelmann et al. 2008). Thus, it is possible that the magnitude and direction of Gadd45β regulation of cell fate varies depending on the cell type as well as on the properties of the stimuli. This degree of complexity in terms the biological function was further elucidated by in vivo studies investigating the role of Gadd45β in the modulation of the immune response. In a study by Liu et al. (2005), Gadd45β was demonstrated to regulate the homeostasis of $CD4^+$ T cells. T cells lacking Gadd45β proliferated faster than wild-type controls and demonstrated increased resistance to activation-induced cell death in vitro (Liu et al. 2005). Furthermore, *gadd45β*$^{-/-}$ mice displayed an exacerbated form of experimental autoimmune encephalomyelitis compared withwild-type controls, likely owing to the Gadd45β antiproliferative and proapoptotic functions in T cell homeostasis (Liu et al. 2005).

12.8.2 XIAP

The inhibitory effects of NF-κB on the JNK cascade have also been associated with the upregulation of the caspase inhibitor XIAP, a member of the inhibitor of apoptosis (IAP) family (Hinds et al. 1999). IAPs are classified in accordance with the existence of one to three baculovirus IAP repeat domains within their structure (Hinds et al. 1999). XIAP contains three baculovirus IAP repeat domains that can bind to and inhibit the activation of caspases 3 and 7 and procaspase 9 (Tang et al. 2001). XIAP has also been reported to be a downstream target of NF-κB, and its induction by TNFα is partially reduced in *relA*$^{-/-}$ MEFs (Tang et al. 2001). Overexpression of XIAP was shown to inhibit TNFα-induced cytotoxicity and diminish activation of JNK in NF-κB-deficient cells (Tang et al. 2001). Furthermore, thymocytes from XIAP-transgenic mice are resistant to apoptosis induced by various triggers (Conte et al. 2001). Apart from its ability to block caspases, XIAP can suppress the JNK cascade, because when overexpressed in RelA null cells, it is capable of interfering with both the caspase-dependent and caspase-independent phases of JNK activation by TNFα (Tang et al. 2001; Papa et al. 2004). However, the significance of XIAP to NF-κB-dependent survival is uncertain as *xiap*$^{-/-}$ mice exhibit no obvious apoptotic phenotype (Harlin et al. 2001) and XIAP ablation in MEFs does not affect the kinetics of JNK induction by TNFα (Kucharczak et al. 2003). The precise mechanisms by which XIAP blocks JNK activation and ultimately PCD are still a matter of debate. The function of XIAP in death-receptor signaling has classically been associated with the presence

of baculovirus IAP repeat domains and the direct inhibitory interaction with effector caspases (Hunter et al. 2007). However, many cIAPs contain a second class of domain, a C_3HC_4 RING finger motif that has recently been shown to possess E3 ligase activity, and is capable of targeting signaling proteins for ubiquitination (Galban and Duckett 2010). In a study by Kaur et al. (2005), the RING domain of XIAP was shown to mediate the polyubiquitination of the MAP3K TAK1, triggering the targeted proteosomal degradation of this kinase (Kaur et al. 2005). Subsequent downregulation of TAK1 protein expression resulted in the inhibition of transforming growth factor β_1 mediated activation of JNK and apoptosis in murine hepatocytes (Kaur et al. 2005). This is a novel mechanism by which XIAP could potentially suppress JNK activation and confer resistance to PCD through the ubiquitin-mediated proteosomal degradation of crucial components of the JNK signaling pathway.

12.8.3 A20

Another target of NF-κB that is seemingly involved in mediating the inhibitory activity of NF-κB on the JNK pathway is the zinc finger protein A20. Initial studies indicated that the NF-κB target, A20, downregulated its own expression (Krikos et al. 1992), indicating that A20 participated in a negative-feedback loop to block NF-κB activation in response to proinflammatory signals in order to attenuate and control inflammatory responses. Several models have validated this early hypothesis (Hymowitz and Wertz 2010). The most robust model involved the generation of A20 null mice, which succumb perinatally to systemic inflammation and multiorgan failure as a result of unchecked NF-κB activity (Lee et al. 2000). Recently, substantial progress has been made in understanding the biochemical and molecular mechanisms by which A20 constitutes a negative-feedback loop to terminate NF-κB activation. A20 possesses dual ubiquitin editing functions for RIP1, a critical signaling molecule in TNFα-mediated NF-κB activation (Wertz et al. 2004). In brief, ubiquitin editing can be broadly conceptualized as the removal of modifications that promote signaling, complex assembly, and activation, such as Lys63–linked polyubiquitination, linear ubiquitin chains, or even ligation with small ubiquitin-like modifier, followed by the addition of modifications that promote substrate degradation, such as Lys11 or Lys48 polyubiquitination, resulting in the attenuation of signaling (Wertz et al. 2004). A20 is capable of deubiquitinating Lys63-linked polyubiquitin chains of RIP1 by its N-terminal ovarian tumor domain and adding Lys48-linked polyubiquitin chains by the C-terminal zinc-finger-containing domain (Coornaert et al. 2009; Renner and Schmitz 2009; Vereecke et al. 2009). In addition to RIP1, A20 has been shown to target other ubiquitinated proteins in the NF-κB signaling pathway, such as TRAF6 and cIAP1 (Boone et al. 2002; Wertz et al. 2004; Shembade and Harhaj 2012).

Given the prosurvival function of NF-κB and the ability of A20 to terminate NF-κB activity, A20 is expected to possess a potent proapoptotic function. This

proapoptotic effect contributes to the tumor suppressor function of A20 in lymphomas, where the reintroduction of wild-type A20 in A20-inactivated lymphoma cells promotes cell death (Kato et al. 2009; Schmitz et al. 2009). However, this effect seems to be cell-type-specific and context-specific, since accumulating evidence suggests the opposite, whereby A20 acts mainly as an antiapoptotic protein, as it protects most cells from TNFα-induced cell death. Accordingly, $a20^{-/-}$ MEFs exhibit persistent JNK activation and exaggerated PCD following exposure to TNFα (Lee et al. 2000; Boone et al. 2002). Additional A20 null cells, such as splenocytes and enterocytes, display enhanced sensitivity to TNFα-induced apoptosis (Lee et al. 2000; Vereecke et al. 2009). In terms of the precise mechanism(s) by which A20 blunts activation of the JNK cascade, RIP1 does not participate in the induction of JNK by TNF-Rs; therefore, the A20-mediated suppression of the JNK pathway is likely to be mediated through a mechanism that does not involve the A20 ubiquitin-mediated inactivation of RIP1 and TNF-R1 signaling complex formation. Indeed, in a recent study by Won et al. (2010), A20 was shown to blunt TNFα-induced JNK activation in cells by reducing the stability and promoting the degradation of ASK1, an important MAP2K in the JNK signaling pathway, through its ubiquitin editing activity (Won et al. 2010). Additional studies are required in order to further elucidate the functional characteristics of A20 in animal pathophysiology.

12.9 NF-κB-Mediated Survival Involves Suppression of ROS

Recent studies have unveiled another mechanism by which NF-κB blocks cell death triggered by TNFα-induced JNK cascade activation that involves the suppression of the accumulation of ROS (Sakon et al. 2003; Pham et al. 2004; Kamata et al. 2005). "Reactive oxygen species" (ROS), which are produced by all types of mammalian cells, is a collective term that includes not only oxygen radicals (superoxide, $O_2^{\cdot -}$, and hydroxyl, OH·) but also some nonradical derivatives of molecular oxygen (O_2), such as hydrogen peroxide (H_2O_2) (Declercq et al. 2009; Morgan and Liu 2010, 2011). Although ROS production is mostly thought to occur under pathological conditions, ROS are continuously generated during normal aerobic metabolism, primarily by the mitochondrial electron transport chain and peroxisomes (Declercq et al. 2009; Morgan and Liu 2010, 2011; Giordano and Terlecky 2012). Leakage of electrons from the electron transport chain results in the formation of the superoxide radical, a moderately reactive species that can generate H_2O_2, which in turn generates highly reactive hydroxyl radicals. Although the mitochondria are by far the greatest source of cellular ROS, extramitochondrial sources of ROS also exist. NAPDH oxidases are a family of enzymes that are dedicated to ROS production (Brown and Griendling 2009). Activated cells of the innate immune system, such as macrophages and neutrophils, activate the phagocytic form of NAPDH oxidase, NOX2 (formerly known as gp91phox), in order to produce superoxide for defense against invading microbial

pathogens. Various types of nonphagocytic cells, including endothelial cells, vascular smooth muscle cells, fibroblasts, and cardiac myocytes, are also known to produce ROS via NADPH oxidases (Brown and Griendling 2009). Additional ROS-generating enzymes include lipoxygenases and cyclooxygenases, which are involved in inflammatory pathways (Declercq et al. 2009; Morgan and Liu 2010, 2011). Peroxisomes are essential cellular organelles that perform important metabolic functions in organisms. Mammalian peroxisomes are densely populated by enzymes that produce ROS, the majority of which are FAD-dependent oxidases—which generate H_2O_2 as a by-product (Giordano and Terlecky 2012). Some other observations indicate the presence of the $O_2^{\cdot -}$-producing enzyme xanthine oxidoreductase (Giordano and Terlecky 2012). Owing to their high reactivity, ROS are a serious hazard for the cell, as they can oxidize macromolecules, thus damaging proteins, lipids, and DNA. ROS accumulation, referred to as oxidative stress, results in cytotoxicity (Declercq et al. 2009; Morgan and Liu 2010, 2011). Hence, cell viability depends on diverse antioxidant systems for ROS detoxification. Under normal conditions, low levels of intracellular ROS are strictly maintained by systems of antioxidant enzymes and their substrates, such as the glutathione and thioredoxin systems, superoxide dismutases, catalase, and peroxiredoxins, as well as other nonenzymatic antioxidants, which collectively scavenge ROS (Declercq et al. 2009; Morgan and Liu 2010, 2011). Although ROS are rapidly eliminated and kept at very low levels, certain extracellular stimuli enhance ROS production, in which case ROS may serve as second messengers in downstream signaling pathways such as the TNFα signaling pathway. Indeed, TNFα has been reported to induce ROS accumulation in a variety of cell types, and these ROS have been shown to be important mediators of PCD (Declercq et al. 2009; Morgan and Liu 2010, 2011).

ROS are potent inducers of JNK in response to TNFα and other stimuli. A substantial body of evidence indicates that NF-κB inhibits JNK activation, and hence PCD, by controlling the accumulation of ROS induced downstream of TNF-Rs and other receptors (Sakon et al. 2003; Pham et al. 2004, Kamata et al. 2005). ROS are key mediators in the death signaling pathways initiated by the triggering of TNF-R1. This physiological role of NF-κB in the restraint of ROS accumulation was initially suggested in studies using NF-κB-deficient cells. Unlike what is seen in normal cells, treatment with TNFα leads to exaggerated accumulation of ROS in NF-κB-deficient cells, indicating that these cells are defective in their ability to maintain redox homeostasis (Sakon et al. 2003; Pham et al. 2004, Kamata et al. 2005). Consistently, exposure to an antioxidant agent such as *N*-acetylcysteine, butylated hydroxyanisole (BHA), or pyrrolidine dithiocarbamate virtually abrogated TNFα-inflicted killing in NF-κB-deficient cells (Sakon et al. 2003; Pham et al. 2004, Kamata et al. 2005). In terms of how ROS accumulation induces PCD, evidence suggests ROS-inflicted cell death is mediated in part by a prolonged activation of the JNK cascade. In *relA*$^{-/-}$ fibroblasts and other NF-κB-deficient cells, sustained activation of JNK signaling downstream of TNF-Rs is abolished by treatment with the ROS neutralizing agents *N*-acetylcysteine and BHA (Sakon et al. 2003; Pham et al. 2004, Kamata et al. 2005). Several studies

have established ROS as a link between the TNFα-induced antiapoptotic NF-κB and proapoptotic JNK pathways both in vitro and in vivo, whereby the attenuation of ROS represents an alternative indirect mechanism through which NF-κB exerts a restraint on sustained activation of the JNK cascade and subsequent induction of PCD. In the mouse model of diethylnitrosamine-induced HCC, specific deletion of IKKβ in hepatocytes resulted in enhanced oxidative stress ROS accumulation after diethylnitrosamine administration, resulting in JNK activation and compensatory proliferation of hepatocytes, with resultant HCC development (Maeda et al. 2005). Administration of the antioxidant BHA reversed the elevation in HCC load caused by IKKβ loss owing to the blockade of prolonged JNK activation and compensatory proliferation. BHA was also shown to protect IKKβ$^{\Delta hep}$ mice from ConA-induced liver failure by elevating ROS accumulation and sustained JNK activation (Kamata et al. 2005). Two important questions that are raised from this study are firstly, how does ROS accumulation lead to prolonged JNK activation? Secondly, how does NF-κB activation prevent ROS accumulation?

12.10 Mechanisms of ROS-Mediated Activation of JNK

In terms of elucidating how ROS accumulation results in prolonged JNK activation, a problem arises in defining ROS targets because ROS have a mixture of roles in cell death. ROS may directly oxidize cellular proteins, lipids, or nucleic acids and therefore cause general cellular damage, or ROS can initiate cell death by acting as either initiators or second messengers in various signaling pathways (Declercq et al. 2009; Morgan and Liu 2010, 2011). Another problem in defining the role of ROS is that they may function at multiple points within a given signaling pathway. Generally, the magnitude and duration of JNK activity is determined by a balance between activating kinases and inhibitory phosphatases, establishing two possible strategies for ROS to enhance JNK activation (Declercq et al. 2009; Morgan and Liu 2010, 2011). In one mechanism, JNK activity is enhanced by the ROS-mediated inactivation of JNK phosphatases (Kamata et al. 2005). A study by Kamata et al. (2005) demonstrated that phosphatases of the MAP kinase phosphatase (MKP) family (MKP-1, MKP-3, MKP-5, and MKP-7), which are known to be involved in downmodulation of MAPK activation, are targets of ROS in the TNFα-induced pathway of JNK activation and PCD (Kamata et al. 2005). TNFα-induced ROS was shown to oxidize critical cysteine residues in the catalytic site of various MKPs, leading to their inactivation and rapid degradation by the ubiquitin–proteasome pathway (Kamata et al. 2005). This ROS-mediated inactivation of MKPs impairs the shutdown of JNK signaling following exposure to TNFα, thus prompting persistent activation of JNK, and ultimately cell death (Kamata et al. 2005). Additionally, studies performed by Matsuzawa et al. (2005) indicate that ROS also trigger the activation of ASK1, a TRAF2-associating MAP3K that is essential for activation of JNK (and p38) downstream of TNF-R1 (Matsuzawa et al. 2005). The reduced form of thioredoxin has been reported to

bind to ASK1, thereby preventing its kinase activity. Following its ROS-induced oxidation, thioredoxin is released and ASK1 is activated (Noguchi et al. 2005). Since *ask1*$^{-/-}$ MEFs are substantially resistant to the sustained JNK activity and apoptosis initiated by ROS such as H_2O_2 (Tobiume et al. 2001), the ROS–thioredoxin–ASK1 axis is believed to be an important molecular switch that may mediate ROS-dependent signaling to JNK, resulting in JNK activation. Taken together, these studies suggest that TNFα-induced ROS manipulate JNK activity by promoting the persistent activation of ASK1 (Tobiume et al. 2001; Matsuzawa et al. 2005; Noguchi et al. 2005) and simultaneously blocking the inhibitory MKPs, thus shifting the balance toward prolonged TNFα-induced JNK activation (Kamata et al. 2005). The relative importance of each mechanism for control of TNFα-induced JNK activity is likely to depend on the cell type and biological context. There is now general consensus that more often than not ROS lie upstream of JNK in the TNF-R-induced pathways of PCD.

12.11 The Cellular Origin of TNFα-Induced ROS

The source of ROS produced downstream of TNF-Rs is still currently a subject of debate. TNFα-stimulated ROS have traditionally been proposed to come from downstream events involving the mitochondria (Declercq et al. 2009; Morgan and Liu 2010, 2011). However, more recently TNFα has been shown to cause the production of superoxide through the activation of NAPDH oxidases. Several lines of evidence have pointed to an involvement of mitochondria-derived ROS in TNFα-induced PCD. Initial studies showed that treatment with TNFα results in damage and dysfunction of the mitochondrial electron transport chain, thereby leading to ROS production (Declercq et al. 2009; Morgan and Liu 2010, 2011). Furthermore, BHA, an antioxidant that accumulates in the mitochondria, protects cells from TNFα-induced PCD (Kamata et al. 2005). Evidence for an extramitochondrial, source of signal-transducing ROS induced in response to TNFα stimulation was presented when ectopic expression of the mitochondrial antioxidant enzyme, manganese superoxide dismutase (Mn-SOD), was shown to afford only limited protection against TNF-R-mediated cytotoxicity in NF-κB null cells (Sakon et al. 2003; Pham et al. 2004). NOX family members are transmembrane proteins that transport electrons across biological membranes to reduce oxygen to superoxide. Most NOX enzymes primarily consist of two NADPH oxidase subunits, NOX2 and p22phox. They form a heterodimeric flavocytochrome b_{-558} that constitutes the catalytic core of the enzyme, but exists in a dormant state in the absence of the other subunits. These additional subunits play mostly regulatory roles ensuring activity and membrane translocation, and are located in the cytosol during the resting state. They include the multidomain proteins p67phox, p47phox, and NOXO1, as well as the small GTPase Rac, which is a member of the Rho family of small GTPases (Brown and Griendling 2009; Morgan and Liu 2010). Numerous reports have demonstrated that TNFα signaling increases transcription of various NADPH

oxidase components, resulting in TNFα-induced oxidase activity. TNFα has also recently been demonstrated to be a direct activator of NAPDH oxidases in various cell types. In L929 cells, NOX1, NOXO1, and Rac1 form a complex with TNF-R signaling components in a TNF-dependent manner, leading to cell death (Kim et al. 2007). One mechanism suggested as a means of NOX1 activation involves the interaction between the TNF-R complex adaptor proteins RIP1 and TRADD with NOXO1. Expression of a dominant negative TRADD with a mutation in its poly-proline eliminated NOXO1 binding and diminished superoxide formation and cell death in TNFα-treated L929 cells (Kim et al. 2007). RIP1 was also shown to be involved in the recruitment of NOX1 and Rac1 to the TNF-R signaling complex (Kim et al. 2007). On the basis of the NOXO1 affinities for RIP1 and TRADD, it has been proposed that RIP1 recruits NOXO1 and the other signaling components (NOX1/NOXA1/Rac1) to the TNF-R complex, where the TRADD–NOXO1 interaction promotes oxidase activation (Kim et al. 2007). Further studies will be required to determine the precise mechanisms involved in ROS production in response to TNFα and other inflammatory stimuli.

12.12 The NF-κB-Mediated Targeting of ROS Controls the JNK Pathway

One of the most significant ways in which NF-κB activity has been shown to influence ROS accumulation is via increased expression of target genes encoding antioxidant proteins, as discussed in the following sections (see Fig. 12.2):

12.12.1 Ferritin Heavy Chain

Through the use of a gene-array-based screen, ferritin heavy chain (FHC) was shown to be a critical mediator of the antioxidant and antiapoptotic activities of NF-κB downstream of TNF-Rs (Pham et al. 2004, Kiessling et al. 2009). Ferritin is a highly conserved and ubiquitously expressed iron storage protein that consists of two subunits, FHC and ferritin light chain, which sequester excess free iron molecules to minimize the generation of iron-catalyzed ROS (Arosio and Levi 2002; Torti and Torti 2002). FHC possesses a ferroxidase activity, which converts toxic Fe^{2+} into nontoxic Fe^{3+}, whereas ferritin light chain has no ferroxidase activity but is likely to contribute to stabilization of assembled ferritin proteins for long-term iron storage (Arosio and Levi 2002; Torti and Torti 2002). In eukaryotes, iron is required both for iron-dependent reactions and for processes such as erythropoiesis, as well as for production of mitochondrial $O_2^{\cdot-}$ and for generating highly reactive ·OH radicals from H_2O_2 (Fenton reaction) (Arosio and Levi 2002; Torti and Torti 2002). Therefore, limiting the availability of this metal, through an

upregulation of ferritin, is a mechanism by which cellular ROS can be controlled. It should be noted, however, that although large amounts of free iron are toxic, a small amount of free cytosolic iron—the so-called labile iron pool—is tolerated by the cell (Vulcano et al. 2000; Arosio and Levi 2002; Pham et al. 2004). Consequently, overexpression of FHC results in the downregulation of the labile iron pool and lower levels of ROS (Picard et al. 1998; Pham et al. 2004).

FHC is induced by TNFα through a mechanism dependent on NF-κB and is required for antagonism of TNFα-induced killing (Pham et al. 2004). FHC overexpression was also demonstrated to counter TNFα-induced apoptosis in NF-κB-deficient cells (Pham et al. 2004). The protective action of FHC against TNFα-inflicted PCD is mediated through iron sequestration, which prevents ROS accumulation and the subsequent sustained activation of JNK downstream of TNF-Rs. The relevance of FHC to the NF-κB-activated mechanism for restraint of TNF-R-induced JNK signaling and PCD has been validated in FHC-deficient cells in in vitro and in vivo systems (Picard et al. 1998; Pham et al. 2004, Kiessling et al. 2009). Indeed, knockdown of FHC in fibroblasts results in persistent activation of JNK by TNFα and hypersensitivity to TNF-R-induced cytotoxicity. The induction of FHC appears to be a critical element for control of the TNFα-induced fluctuations of the intracellular labile iron pool (Picard et al. 1998; Pham et al. 2004, Xie et al. 2005; Kiessling et al. 2009). Significantly, systemic administration of the iron chelator deferoxamine has been reported to protect mice against TNF-R-mediated lethality and tissue damage, thereby suggesting the relevance of this mechanism in vivo (Vulcano et al. 2000; Xie et al. 2005).

An elevation of ROS level is often required for oncogene-driven transformation (Bernard et al. 2001; Torti and Torti 2002). On the other hand, this elevation can also enhance the propensity of certain malignant cells to undergo cell death. On balance, accumulating evidence suggests that FHC plays an important role in cancer. For instance, increased expression of FHC has been observed in a colon cancer cell line that showed a highly malignant phenotype in vivo (Modjtahedi et al. 1992). Increased ferritin concentration in tumor versus normal tissue has also been shown in several human malignancies, such as colon cancer (Vaughn et al. 1987), breast cancer (Weinstein et al. 1982), seminoma (Cohen et al. 1984), and renal cell carcinoma (Partin et al. 1995). In a recent study on cutaneous T cell lymphoma, inhibition of the NF-κB pathway by specific inhibitors caused iron- and ROS-dependent, but caspase- and cathepsin-independent, cell death of cutaneous T cell lymphoma cell lines and of primary cells from patients with Sézary syndrome, a leukemic variant of cutaneous T cell lymphoma (Kiessling et al. 2009). The induction of oxidative stress and cell death following the inhibition of NF-κB in these cells was ablated by deferoxamine treatment (Kiessling et al. 2009). Moreover, the use of a murine T cell lymphoma model demonstrated that NF-κB inhibition significantly delays tumor growth in vivo by downregulating FHC (Kiessling et al. 2009). Collectively, this evidence suggests that the upregulation of FHC is a means by which NF-κB promotes oncogenesis and cancer progression (Weinstein et al. 1982; Cohen et al. 1984; Vaughn et al. 1987; Modjtahedi et al. 1992; Partin et al. 1995; Kiessling et al. 2009).

12.12.2 Mn-SOD

NF-κB antioxidant activity has also been associated with the upregulation of the ROS scavenger Mn-SOD (Bernard et al. 2001; Delhalle et al. 2002; Bubici et al. 2004). Mn-SOD is a key antioxidant enzyme located in the mitochondrial matrix that protects cells from oxidative stress by catalyzing the dismutation of $\cdot O_2^-$ to H_2O_2 (Curtin et al. 2002). Mn-SOD is a TNFα-inducible NF-κB target, and when overexpressed, has been demonstrated to attenuate TNFα-mediated cytotoxicity in certain systems (Bernard et al. 2001; Delhalle et al. 2002; Pham et al. 2004). Furthermore, mice lacking Mn-SOD die shortly after birth owing to massive oxidative stress (Li et al. 1995). However, the significance of Mn-SOD for the pro-survival action of NF-κB remains uncertain owing to the observations that in various NF-κB-deficient systems, ectopic expression of Mn-SOD affords little or no protection against TNFα-induced PCD (Siemankowski et al. 1999; Nakashima et al. 2003; Sakon et al. 2003; Pham et al. 2004). Nevertheless, Mn-SOD may be a critical effector of the antioxidant and protective activities in specific biological contexts. Indeed, Mn-SOD was shown to be essential for radiation resistance in cancer cells (Hirose et al. 1993). Sun et al. (1998) demonstrated that Mn-SOD overexpression protects Chinese hamster ovary cell lines from cell death induced by ionizing radiation (Sun et al. 1998). Furthermore, Josson et al. (2005) found that RelB nuclear localization and Mn-SOD promoter binding is increased following exposure of the aggressive PC-3 prostate cancer cell line to ionizing radiation. Inhibition of RelB activity in PC-3 cells by RNA interference of overexpression of dominant negative p100 mutant results in a decrease in Mn-SOD expression and an increase in radiation sensitivity (Josson et al. 2006).

It is possible that for effective control of ROS levels, the synergistic activities of FHC and Mn-SOD are potentially crucial. Whereas induction of Mn-SOD promotes dismutation of O_2^- into H_2O_2, FHC-mediated iron sequestration may facilitate disposal of H_2O_2 by peroxidases and catalases (Curtin et al. 2002; Torti and Torti 2002). In NF-κB-deficient cells, FHC levels are usually low (Pham et al. 2004), and as a consequence, free iron remains available to catalyze the Fenton reaction reducing H_2O_2 to highly reactive $\cdot OH$ radicals (Arosio and Levi 2002; Torti and Torti 2002). This could explain the inability of ectopic Mn-SOD to inhibit, alone, TNFα-induced cytotoxicity in NF-κB null cells. Further studies will be required to clarify this issue (Delhalle et al. 2002; Sakon et al. 2003; Pham et al. 2004).

12.13 Concluding Remarks

In recent years, the search for cancer therapies has focused on targeting specific signaling pathways that drive inappropriate cell growth and survival, thereby offering the promise of greater specificity coupled with reduced systemic toxicity. The pivotal importance of the NF-κB pathway in human diseases has resulted

in a surge in interest in developing specific inhibitors of the NF-κB pathway for treating cancer and other disorders. However, NF-κB-targeting drugs have failed to produce the intended results in patients. Proteasome inhibitors (e.g., bortezomib), for instance, which block the proteolysis of IκBs and are indicated in multiple myeloma, are dose-limiting and ineffective in achieving long-lasting disease remission in cancer patients because of low therapeutic indices and adverse side effects owing to the pleiotropic functions of NF-κB and the proteasome (Baud and Karin 2009). Serious misgivings also exist for IKK inhibitors, which have yet to be clinically approved because of safety concerns (Greten et al. 2007; Baud and Karin 2009; DiDonato et al. 2012). These safety concerns arise from the pronounced ability of IKK inhibitors to enhance the production of IL-1β and related cytokines (Greten et al. 2007). A preferable therapeutic approach would be to develop drugs that target the critical downstream NF-κB-regulated genes that promote survival in cancer and link this with inflammation rather than NF-κB itself. The discovery that the suppression of ROS and JNK signaling is a key protective mechanism mediated by NF-κB now affords an opportunity to develop such drugs. Small-molecule inhibitors that block Gadd45β binding to MKK7 or inhibit the antioxidant proteins induced by NF-κB potentially could result in an elevation of JNK and ROS cytotoxic signaling, thereby blocking cell proliferation and inducing cell death in cancerous and proinflammatory cells. Such strategies could potentially make possible the selective blockade of the prosurvival action of NF-κB in cancer or specific inflammatory cells, without significantly compromising the ability of NF-κB to serve in immunity and other functions. Additionally, since NF-κB, JNK, and ROS activities are likely to be predominantly upregulated in inflamed and cancerous tissues, therapeutic strategies aimed at inferring with the interplay between these activities are likely to have inherent specificity for diseased tissues. Such selective blockade could help limit the inherent toxicity of drugs that target core components of the NF-κB pathway.

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Part III
Functions and Dysfunctions of Physiological Processes Involved in Tumor Genesis and Malignancy

Chapter 13
Migration, Metastasis, and More: The Role of Chemokines in the Proliferation, Spreading, and Metastasis of Tumors

Rolf Mentlein, Kirsten Hattermann and Janka Held-Feindt

Abstract Chemokines are a family of small peptides (8–15 kDa) that were first discovered as cytokines with chemotactic properties. Subsequently, it became clear that chemokines have much broader functions in tissue development and homeostasis as well as in pathological responses, especially in inflammation and cancer. After providing a short overview of chemokines, their regulation, receptors, and signal transduction, we outlines their role in various steps of tumor progression, in particular with regard to tumor cells, tumor stem cells, the tumor microenvironment, tumor angiogenesis, and the spreading/metastasis of tumors. The focus is on CXC chemokines and selected tumor types, e.g., gliomas, melanomas, and breast cancer. In addition to the tumor-promoting functions of chemokines and their receptors in the different steps of cancerogenesis, their potential as diagnostic and therapeutic targets is discussed.

Keywords Angiogenesis • Chemotaxis • Proliferation • Metastasis • Cancer

13.1 Structural Biology of Chemokines and Their Receptors

13.1.1 Structure of Chemokines

Chemokines are a family of small cell-secreted peptides of about 8–10 kDa. Their name is derived from their ability to induce directed chemotaxis in nearby responsive cells; they are *chemo*tactic cyto*kines*. They share four conserved cysteine

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residues that are key for their three-dimensional structure and for their division into four subgroups (Fig. 13.1).

Chemokines are found in all vertebrates, some viruses, and a few bacteria, but none have been detected in invertebrates. In humans, about 50 chemokines exist, and 23 receptors are known (Table 13.1). Typical chemokines are produced as propeptides with signal sequences of about 20 amino acids that are liberated by the cellular signal peptidase to from the active (mature) chemokine, which then is constantly secreted from the cell. Many chemokines are tightly regulated in their transcription and subsequent secretion by cytokines and other growth factors, others are produced constitutively.

A further regulation, activation or inactivation, of chemokine activities occurs by posttranslational processing by cell-surface or extracellular proteases (Wolf et al. 2008; Mortier et al. 2011). The endoproteases matrix metalloproteinases, cathepsins B, D, and L, and plasmin/plasmin activators (urokinase) as well as exopeptidases such as dipeptidyl peptidase IV (CD26) and carboxypeptidase N play a decisive role in this regulation. Destruction of the N-terminus (e.g., by dipeptidyl peptidases) destroys chemokine activity, but binding occurs also through other domains; therefore, these N-terminal truncated chemokines can act as antagonists (Ludwig et al. 2002; Mentlein 2004).

Among the chemokines, two exhibit a unique transmembrane structure: CXCL16 and CX3CL1. Both are synthesized in a transmembrane pro-form from which the soluble chemokine is liberated by the action of cell-surface proteases, namely, by sheddases (Fig. 13.2). As such sheddases, ADAM10 and ADAM17 ("ADAM" is an acronym for "a disintegrin and metalloproteinase") have been

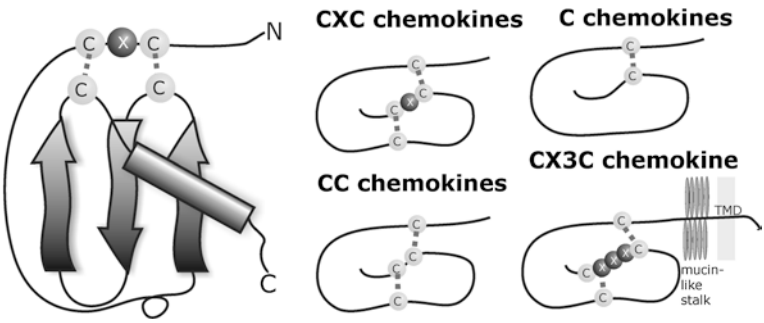

Fig. 13.1 Chemokines are subdivided into four groups according to the structural location of cysteine residues, which either directly neighbor (CC chemokines or β-chemokines) or are separated by one (CXC chemokines or α-chemokines) or three (CX3C chemokines or δ-chemokines, only CX3CL1, also known as is fractalkine or neurotactin) amino acids; C chemokines, or γ-chemokines, have only two cysteine residues (XCL1, also known as lymphotactin-α, and XCL2, also known as lymphotactin-β). Their three-dimensional structure (*left*) includes the cysteine bridges at the N-terminus followed by a loop of approximately ten amino acids (N-loop), a single-turn helix, called a 3_{10}-helix, three β-strands, and a C-terminal α-helix. *TMD* transmembrane domain

Table 13.1 Chemokines, synonyms, and receptors

Chemokine	Synonym	Receptors
CXC/α		
CXCL1	GRO-α, MGSA-α	CXCR2
CXCL2	GRO-β, MGSA-β	CXCR2
CXCL3	GRO-γ, MGSA-γ	CXCR2
CXCL4	PF-4	CXCR3B
CXCL5	ENA-78	CXCR2
CXCL6	GCP-2	CXCR1, CXCR2
CXCL7	PPBP, NAP-2	CXCR1, CXCR2
CXCL8	IL-8	CXCR1, CXCR2
CXCL9	MIG	CXCR3A, CXCR3B
CXCL10	γIP-10	CXCR3A, CXCR3B
CXCL11	I-TAC	CXCR3A, CXCR3B, CXCR7
CXCL12	SDF-1α	CXCR4, CXCR7
CXCL13	BCA-1	CXCR5
CXCL14	BRAK	Unknown
CXCL15 (Mm)	Lungkine	Unknown
CXCL16	SR-PSOX	CXCR6
CXCL17	VCC-1, DMC	Unknown
CC/β		
CCL1	I-309	CCR8
CCL2	MCP-1	CCR2
CCL3	MIP-1α, LD78	CCR1, CCR5
CCL3L1/CCL3L3	LD78β	CCR5
CCL3L2	LD78γ	Truncated pseudogene
CCL4	MIP-1β, LAG-1	CCR5
CCL4L1	LAG-1 gene duplication	CCR5
CCL5	RANTES	CCR1, CCR2, CCR3
CCL6 (Mm, Rn)	C10	CCR1, CCR2, CCR3
CCL7	MCP-3	CCR1, CCR2, CCR3
CCL8	MCP-2	CCR1, CCR2, CCR5
CCL9/CCL10 (Mm, Rn)	MRP-2, CCF18	CCR1
CCL11	Eotaxin	CCR3
CCL12 (Mm, Rn)	MCP-5	CCR2
CCL13	MCP-4	CCR1, CCR2, CCR3, CCR5
CCL14	HCC-1	CCR1, CCR5
CCL15	HCC-2, MIP-5, Lkn-1	CCR1, CCR3
CCL16	HCC-4, LEC, MTN-1	CCR1, CCR2, CCR5
CCL17	TARC	CCR4
CCL18	DC-CK-1, PARC, MIP-4	Unknown
CCL19	ELC, MIP-3β	CCR7, CCRL1
CCL20	LARC, MIP-3α	CCR6
CCL21	6Ckine, SLC	CCR7, CCRL1
CCL22	MDC	CCR4
CCL23	MPIF-1, MIP-3	CCR1
CCL24	MPIF-2, eotaxin-2	CCR3

(contined)

Table 13.1 (continued)

Chemokine	Synonym	Receptors
CCL25	TECK	CCR9, CCRL1
CCL26	MIP-4α, eotaxin-3	CCR3
CCL27	ESkine, CTACK	CCR10
CCL28	MEC, CCK1	CCR3, CCR10
C/γ		
XCL1	Lymphotactin-α	XCR1
XCL2	Lymphotactin-β	XCR1
CX3C/δ		
CX3CL1	Fractalkine, neurotactin	CX3CR1

Mm Mus musculus, *Rn Rattus norvegicus*

unambiguously identified (Abel et al. 2004; Ludwig et al. 2005; Ludwig and Mentlein 2008). However, the transmembrane forms also have biological activities, at least as adhesion molecules (Fig. 13.2).

Besides proteolytic modification, the availability of some active chemokines depends on their binding to cell-surface or extracellular glycosaminoglycans (Allen et al. 2007; Rot 2010). Furthermore, some chemokines oligomerize by themselves (Ray et al. 2012) or oligomerize by glycosaminoglycan binding, and this can regulate their actions. Several chemokines also heterodimerize with others, but the relevance of this has not been demonstrated.

13.1.2 Chemokine Receptors and Their Signal Transduction

Chemokines exert their biological effects by interacting with G-protein-linked, seven-transmembrane domain receptors (Fig. 13.3) that are found on the surfaces of their target cells. Since only 23 chemokine receptors exist, but many more chemokine ligands are known, most receptors bind several chemokines (Table 13.1). However, a single chemokine might also target several receptors.

Chemokine receptors are composed of about 340–370 amino acids with a short, acidic N-terminal extracellular domain, seven helical transmembrane domains plus three intracellular and three extracellular hydrophilic loops, and an intracellular C-terminus containing serine and threonine residues that act as phosphorylation sites during receptor regulation (Allen et al. 2007). A DRY motif (DRYLAIV or similar sequence) in the second intracellular loop is supposed to be essential for G-protein-mediated signaling (Fig. 13.3). Receptors lacking this motif might bind chemokines without signaling and are therefore supposed to be decoy (or scavenger) receptors that regulate chemokine levels by binding and internalization. However, recently it has been shown that at least CXCR7 can signal via β-arrestin (Rajagopal et al. 2010; Ödemis et al. 2012). Signaling is further complicated by the formation of receptor homo-oligomers and hetero-oligomers (Allen et al. 2007).

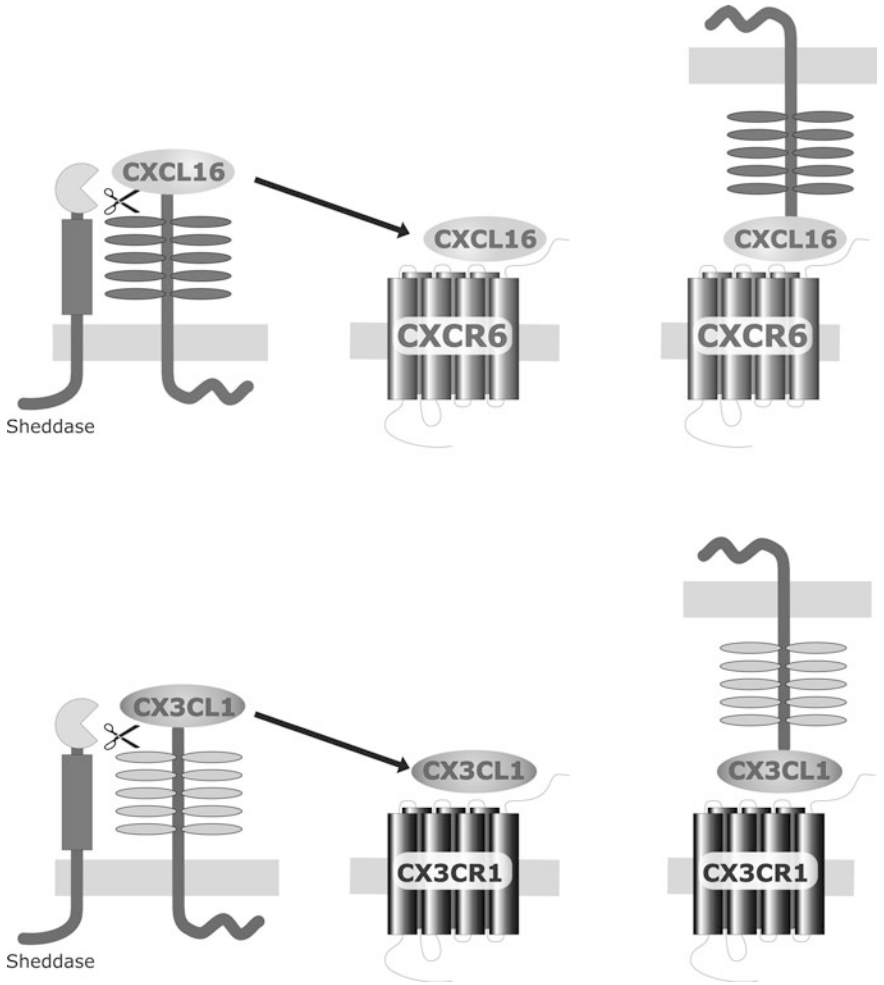

Fig. 13.2 The transmembrane chemokines CXCL16 and CX3CL1 are exceptional in structure: they are synthesized in a cell-surface membrane-bound form with a C-terminal, extracellular chemokine domain, a highly glycosylated extracellular stalk, a transmembrane region, and a short intracellular domain. Cleavage by sheddases (mostly cell-surface proteases) liberates soluble, chemotactic chemokines . The transmembrane forms, by interaction with their receptors, can at least serve as cell adhesion molecules

On ligand binding, chemokine receptors activate their G proteins, and the Gα and Gβγ subunits dissociate and activate downstream effectors. Receptor desensitization and internalization is achieved by agonist-dependent phosphorylation of the C-terminal tail which promotes binding of β-arrestins and internalization through clathrin-coated pits or lipid rafts/caveolae. G-protein-dependent chemokine effects can be inhibited by *Bordatella pertussis* toxin. As mostly G_i

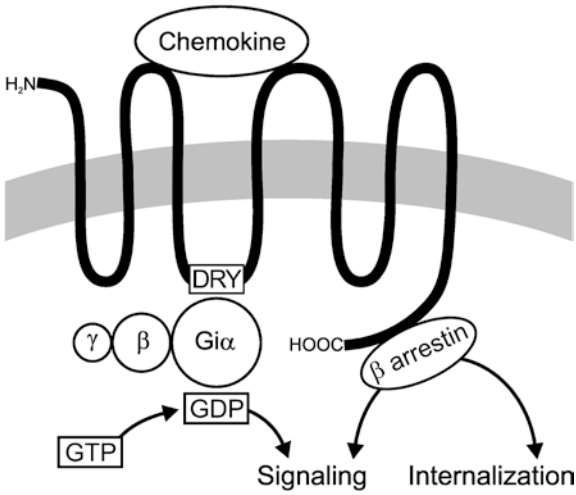

Fig. 13.3 Chemokine receptors and signal transduction. Chemokine receptors are seven-transmembrane receptors that couple to G proteins. The DRY motif (DRYLAIV or similar sequence) in the second intracellular loop is supposed to be essential for G-protein-mediated signaling, and the C-terminal intracellular part is supposed to be essential for β-arrestin-mediated internalization. However, signaling via β-arrestin can also occur

proteins are bound, signal transduction often involves inhibition of adenylate cyclase, activation of phospholipase C with generation of inositol trisphosphate/Ca^{2+} and diacylglycerol as second messengers, and activation of several kinases.

13.1.3 Biology of Chemokines

The initially detected targets of chemokines are leukocytes. Because cell motility is essential for their functions, chemokines play a dominant role in their physiology by controlling their traffic under basal and inflammatory conditions, but they also control leukocyte adhesion, proliferation, survival, and gene transcription. However, the actions of chemokines are not restricted to leukocytes and inflammation. In addition to being found on leukocytes, chemokine receptors are found on many other cell types, including endothelial and smooth muscle cells, fibroblasts, and stromal and epithelial cells. Furthermore, their function is not restricted to inflammation. They are essentially involved in the development of some tissues, their homeostasis, and in several other diseases (Raman et al. 2011).

A good example of the role of chemokines in tissue development is the brain. Here, chemokines control neurogenesis and gliogenesis and the lamination of neurons (Ransohoff 2009). For example, CXCL12/stromal-cell-derived factor 1 (SDF-1)—CXCR4/CXCR7 interactions regulate movement and laminar positioning of neurons/neural precursors in the cerebellum or cortex (Reiβ et al. 2002; Borrell and Márin 2006). Here, CXCL12 is constitutively produced by meningeal cells, and receptor expression is regulated on neural precursors. CXCL12 is one of the most "ancient" chemokines , and the regulation of migration and development of stem cells also in other tissues suggests that the original function of chemokines

is not necessarily linked to immunity (Huising et al. 2003). Another chemokine highly expressed in the nervous system is CX3CL1 (also termed fractalkine or neurotactin), which is produced by neurons and targets microglia cexpressing the receptor CX3CR1. This neuron–microglia axis appears to be a major regulator of microglial neurotoxicity in neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Cardona et al. 2006; Ludwig and Mentlein 2008; Fuhrmann et al. 2010).

As general effects, chemokines control not only cell migration (as their name suggests), but also cell proliferation, cell survival (antiapoptosis), and regulation of gene expression, including cytokine and protease gene expression—all events that are essential parts of tumor formation and progression.

13.2 Chemokines in Tumors

Many chemokines and receptors have been described in tumors of different types (examples are given in Table 13.2), and a detailed description (see Kruizinga et al. 2009) is beyond the scope of this article, which focuses on the principles of chemokine action in solid human tumors and exemplifies this with some selected examples.

In the context of the hallmarks of carcinogenesis outlined by Hanahan and Weinberg (2011), chemokines are fundamentally involved in cancer cell proliferative signaling, resistance to cell death, induction of tumor angiogenesis, activation of invasion, transmigration, and metastasis (Fig. 13.4). Hereby, they target cancer

Table 13.2 Examples of chemokine receptors in different types of solid tumors

Cancer type	Receptors expressed	References
Breast cancer	CXCR4, CXCR7, CCR5, CCR7	Müller et al. (2001), Li et al.(2004), Burns et al. (2006)
Ovarian cancer	CXCR4	Scotton et al. (2001)
Prostate cancer	CXCR4, CX3CR1	Darash-Yahana et al. (2009)
Esophageal cancer	CXCR4	Kaifi et al. (2005)
Stomach cancer	CCR7	Mashino et al. (2002)
Pancreatic cancer	CXCR4	Marchesi et al. (2008)
Colon cancer	CXCR3, CXCR4, CCR7	Kawada et al. (2007), Kim et al. (2005), Li et al. (2011)
Lung cancer	CXCR4, CXCR7, CCR7	Phillips et al. (2003), Miao et al. (2007)
Melanoma	CXCR1–CXCR4, CXCR7, CCR7, CCR9, CCR10	Müller et al. (2001), Letsch et al. (2004)
Neck and head cancer	CXCR4, CXCR5, CCR7	Muller et al. (2006), Katayama et al. (2005)
Neuroblastoma	CXCR4	Russell et al. (2004)
Glioblastoma	CXCR4, CXCR7, CX3CR1	Hattermann et al. (2010), Held-Feindt et al. (2010)

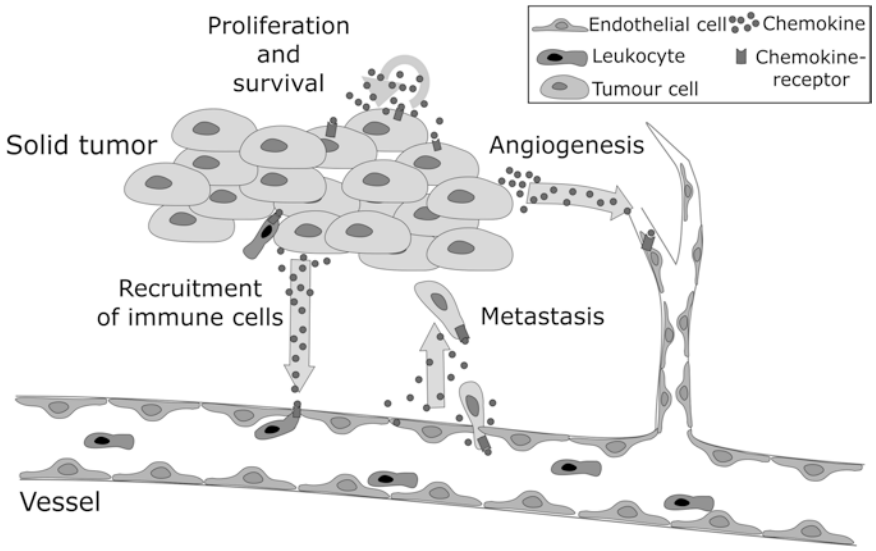

Fig. 13.4 Multiple roles of chemokines in the progression of cancers. Chemokines can directly target tumor cells and increase their proliferation and survival, they can target the tumor stroma, e.g., by stimulation of angiogenesis, or they may recruit immune cells with tumor-promoting or tumor-inhibiting properties to the tumor. As result of increased mobility and invasion, tumor cells may also metastasize to sites of chemokine production in peripheral tissue

cells, promote cancer stem cells, and interact with or are produced by tumor stromal cells such as endothelial cells, pericytes, fibroblasts, or immune inflammatory cells.

13.2.1 Chemokines as Tumor Cell Promoters

Receptors for chemokines are often found on tumor cells, whereas the ligands are produced by the malignant cells themselves, by tumor stromal cells, or by distant cells in sites of metastasis. Chemokine receptors on tumor cells can mediate different responses that favor tumor cell growth, including cell proliferation, resistance to apoptosis, induction of genes enhancing invasion, angiogenesis, and metastasis.

A good example of these tumor-promoting functions is given by the chemokine CXCL12/SDF-1, which targets the receptors CXCR4/fusin/CD184 and CXCR7/RDC1 (Duda et al. 2011). CXCL12 is a homeostatic chemokine that is produced in many cells, including bone marrow stromal cells, fibroblasts,

hepatocytes, glial cells, and meningeal cells. The isoforms SDF-1α and SDF-1β result from alternative splicing and differ in an additional four amino acids at the C-terminus; apparently, they do not differ or differ only slightly in their biological activities, which critically depend on an intact N-terminus.

The originally detected signaling receptor CXCR4 is expressed on stem cells, hematopoietic cells, leukocytes, endothelial cells, and many types of tumor cells. CXCR4 expression is upregulated by many growth factors and cytokines and also by hypoxia—factors/conditions that are found inside many tumors. CXCR7 was cloned as an orphan receptor RDC1 and much later identified as a receptor for CXCL12 and—with tenfold lower affinity—for CXCL11/interferon-inducible T-cell α chemoattractant (I-TAC) (Burns et al. 2006). Because CXCR7 lacks a DRY domain that binds G proteins, it was been regarded as a decoy receptor that regulates CXCL12 levels, but recent studies have shown that CXCR7 can signal via an alternative way, namely, via β-arrestins, which are also involved in internalization of G-protein-coupled receptors (Rajagopal et al. 2010). CXCR4 and CXCR7 are expressed alone or in combination on the surface of various tumor cells. Furthermore, both receptors are expressed on the tumor vasculature (Miao et al. 2007) and on some types of tumor stromal cells, e.g., monocytes/macrophages (Hattermann et al. 2010).

Glioma cells are an interesting example of the effects of CXCL12 on tumor cells via one or another receptor. Gliomas are the commonest type of primary brain tumors and are derived from glial cells (or corresponding stem cells). Often, they have a poor prognosis, especially the most malignant and most frequent form, glioblastoma (or glioblastoma multiforme; WHO grade IV). The ligand CXCL12 is produced within the tumor by malignant cells, but also by palisading astrocytes and microglial and endothelial cells within the tumor. Malignant cells of this aggressive brain tumor express in situ mostly or after culture in medium containing fetal calf serum almost exclusively only CXCR7, whereas tumor stem-like cells express preferentially CXCR4 (Hattermann et al. 2010). This receptor switch can also be mimicked in vitro by addition of fetal calf serum or differentiation factors. Whereas CXCR4-positive glioma cells respond to CXCL12 with increased proliferation and migration, CXCR7-positive cells are protected from the influence of CXCL12 by chemotherapeutic-induced apoptosis—reflecting also a different signal transduction (Fig. 13.5). Thus, CXCL12 promotes tumors in glioma cells directly via two types of receptors that initiate two different mechanisms: tumor expansion and invasion (CXCR4) and tumor survival (CXCR7). Furthermore, this example shows that chemokines can promote tumor progression at different stages with different mechanisms: by switching their receptors and reactions.

Glioma cells are an example of tumor cells where both CXCL12 receptors are expressed separately. However, in other tumor cells (e.g., mammary carcinoma cells), but also on tumor stromal cells (macrophages) and tumor endothelial cells both receptors are expressed together. Thus, they might regulate each other, heterodimerize, or react to CXCL12 stimulation with separate or common pathways.

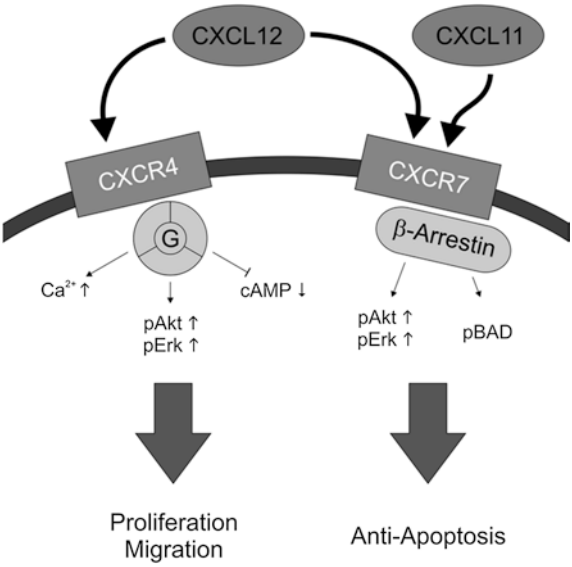

Fig. 13.5 Tumor-promoting effects of a single chemokine via two separate receptors. In human glioblastomas, CXCR4 is expressed on tumor stem cells, where it mediates proliferation and migration, whereas CXCR7 is found on "differentiated" tumor cells, where it promotes tumor survival

13.2.2 Chemokines and Tumor Angiogenesis

Angiogenesis, the sprouting of new blood vessels from existing ones, is a key event in growth and progression of solid tumors. Namely, without an efficient blood supply, tumors cannot grow beyond a critical size of a few cubic millimeters. Tumor cells promote angiogenesis early in tumorigenesis by production of angiogenic factors (Mentlein and Held-Feindt 2003). This "angiogenic switch" is triggered by environmental signals and genetic factors such as metabolic or mechanical stress, lack of oxygen, low pH, hypoglycemia, inflammatory response by infiltrating immune or tumor stromal cells, and genetic mutations (e.g., activation of oncogenes or deletion of tumor-suppressor genes). Perhaps the most important diffusible angiogenic factor is vascular endothelial cell growth factor (VEGF)-A, a glycoprotein of about 45 kDa consisting of two identical peptide chains linked by disulfide bridges. Several other growth and chemotactic factors for endothelial cells have been described, some more endothelial-cell-specific (like VEGF), and others such as basic fibroblast growth factor act "pleiotropically" on several other cell types.

Chemokines also belong to such "pleiotropic" angiogenic factors. Especially CXC chemokines with a conserved Glu-Leu-Arg sequence (ELR motif) at the N-terminus have a potent angiogenic activity. These ELR-positive chemokines include CXCL1 to CXCL8, with the exception of CXCL4. These chemokines act via a common receptor, CXCR2, which is expressed on endothelial cells and mediates the angiogenic activity. The importance of ELR-positive chemokines in supporting angiogenesis during neoplastic progression has been established in a variety of models, including melanomas and prostate, ovarian, and non-small-cell

lung cancer (Strieter et al. 2006). Furthermore, CX3CL1 has been shown to mediate angiogenesis under physiological as well as pathological conditions (Gerber et al. 2009).

Opposite to the angiogenic ELR-positive chemokines, some non-ELR chemokines (CXCL4, CXCL9, CXCL10, CXCL11, and CXCL14) exhibit angiostatic properties (Strieter et al. 2006). Therefore, the balance of ELR-positive versus non-ELR chemokines has been supposed to determine tumor angiogenesis and consequent tumor progression. But also the non-ERL-chemokine CXCL12 acts via CXCR4 on endothelial cells and thereby promotes angiogenesis in vitro (Bachelder et al. 2002). However, in vivo this angiogenic effect of CXCL12–CXCR4 seems to be of less importance. Namely, when animals with breast or non-small-cell lung cancer tumors were treated with neutralizing anti-CXCL12 or anti-CXCR4 antibodies, there was no change in the size of the primary tumors nor was there any evidence for a decline in primary-tumor-associated angiogenesis (Phillips et al. 2003); however, there was a marked attenuation of tumor metastases in an organ-specific manner (Müller et al. 2001).

Overall, chemokines are implicated in tumor angiogenesis in a complex way that may critically depend on the tumor entity and the tumor microenvironment that produce some of these angiogenic (or antiangiogenic) chemokines.

13.2.3 Chemokines in Tumor Inflammation

Cancer is often associated with inflammation, and inflammation is a major cofactor in the pathogenesis of some types of cancer (Ben-Neriah and Karin 2011). Perhaps the most important matchmaker of inflammation is the transcription factor nuclear factor κB, which controls the transcription of many inducible chemokines. These inflammation-induced chemokines recruit distinct leukocyte subsets, promote angiogenesis, or are involved directly or indirectly in tumor cell progression and metastasis. However, depending on the leukocyte subset attracted, tumor-suppressing functions of inflammatory chemokines can also be expected and are known (Allavena et al. 2011).

There is substantial clinical and experimental evidence that tumor-associated macrophages (TAMs) promote cancer initiation and malignant progression (Qian and Pollard 2010). The tumor-promoting functions include support of tumor angiogenesis, tumor cell invasion, migration, intravasation, and immunosuppression. TAMs play a major role in tumor initiation when inflammation is a causal factor.

CC chemokines are major chemoattractants of monocytic precursors circulating in the blood. For example, CCL2 and CCL5 are produced in many types of solid human tumors, and their levels are correlated with high numbers of TAMs (Allavena et al. 2011). But other chemokines can also be implicated in TAM infiltration, e.g., CX3CL1 is produced in large amounts by glioma cells and attracts blood monocytes and brain microglial cells. Additionally, the glioma-infiltrating macrophages/microglial cells are characterized by particularly high expression

of the receptor CX3CR1 (Held-Feindt et al. 2010). Although they are potentially toxic to tumor cells, TAMs display mainly tumor-supporting functions (Qian et al. 2010). This includes promotion of tumor angiogenesis, e.g., by production of VEGF, basic fibroblast growth factor, and angiogenic chemokines, tumor matrix remodeling, e.g., by synthesis of extracellular-matrix-degrading proteases, and immune suppression, e.g., by production of immunosuppressive cytokines/chemokines such as transforming growth factor β or CCL17 and CCL22. Besides tumor growth and invasion, TAMs might also facilitate tumor metastasis. For example, tumor-produced CCL2 recruits inflammatory monocytes bearing its receptor CCR2 and thereby facilitates breast tumor metastasis (Qian et al. 2011). However, tumor cells can also dampen the recruitment of monocytes; tumor-derived matrix metalloproteinases can cleave CC chemokines such as CCL7, yielding a potent antagonist for CCR1, CCR2, and CCR3 (McQuibban et al. 2000).

In many solid tumors, tumor-infiltrating lymphocytes (TILs) are the major leukocyte subset in the tumor stroma, and can mediate an immunosuppressive effect on tumors. In fact, their density and location can predict the clinical outcome (Galon et al. 2006). One important chemokine receptor in TILs is CXCR3. The corresponding ligands CXCL9, CXCL10, and CXCL11 are interferon-inducible, and thus their expression by tumor and tumor stromal cells can be favored by tumor-accompanying inflammation processes leading to infiltration of CD4 and CD8 lymphocytes and antitumoral responses (Pan et al. 2006). On the other hand, tumor cells can also express CXCR3, and this tumor cell expression is often associated with poor survival (Ma et al. 2009). Another chemokine highly produced by tumor and tumor stromal cells is CXCL16 (Ludwig et al. 2005; Held-Feindt et al. 2008; Darash-Yahana et al. 2009), which attracts via its only known receptor CXCR6/Bonzo activated T and natural killer cells. However, some (subsets of) tumor cells can also express the receptor. Since in different types of cancer high CXCL16 expression can been correlated with either good (Hojo et al. 2007; Gutwein et al. 2009) or poor (Darash-Yahana et al. 2009; Matsushita et al. 2011; Ha et al. 2011) prognosis, this may reflect whether immunosuppression or auto/paracrine tumor cell stimulation by this chemokine is dominant.

These examples show the sometime double-edged role of TIL-targeting chemokines: on the one hand, attracting leukocytes that kill tumor cells, and on the other hand, an autocrine role in the growth or spreading of tumor cells themselves.

13.2.4 Chemokines in Tumor Invasion and Metastasis

Metastasis is the process through which cancer cells spread from the initial tumor and migrate to another nonadjacent organ via the bloodstream or the lymphatic system. Metastasis is a complex and multistep process which involves dissemination from the primary tumor, invasion through the surrounding extracellular matrix and vessels, stability and spreading in the circulation, migration through the

endothelial barrier, and finally survival and growth in the target organ. The preference of certain tumors to metastasize in particular organs was first explained by Paget (1889) in the "seed and soil" theory, meaning that it is difficult for cancer cells to survive outside their region of origin, so in order to metastasize they must find a location with similar characteristics. For example, breast tumor cells, which gather calcium ions from breast milk, metastasize to bone tissue, where they can gather calcium ions from bone. Malignant melanoma spreads to the brain, presumably because neural tissue and melanocytes both arise from neural crest cells in the embryo. Later, it was proposed that metastasis occurs purely by anatomic and mechanical routes (Ewing 1928). Today, the "seed and soil" theory has gained new attention from a novel viewpoint (Hart 1982), and chemokines can be now regarded as major players in this process. Namely, chemokines act as chemotactic signals that lead tumor cells to "fertile" grounds and then help them—in concert with other growth factors—to survive (Ben-Baruch 2008; Zlotnik et al. 2011). Furthermore, since chemokines are also involved in the transmigration of leukocytes through endothelial barriers, this might also be a task in tumor metastasis at certain sites, e.g., the brain with its special endothelial barrier.

Generally, evidence for the involvement of chemokines and their receptors in tumor metastasis can be obtained by two approaches: (1) in human patients their occurrence in the primary and metastatic tumor can be compared, and (2) animal experiments can directly monitor their involvement in tumor metastasis.

The first insights for involvement of chemokines in metastasis came from the CXCL12–CXCR4 axis in breast cancer and leukemia cells. Breast cancer typically spreads to regional lymph nodes, lung, liver, and/or bone marrow, sites where CXCL12, the ligand for CXCR4 and CXCR7, is highly expressed. In fact, human breast cancer cells express selected chemokine receptors, among them CXCR4 and CCR7 (Müller et al. 2001). Furthermore, in an orthotopic mouse model, inhibition of the CXCL12–CXCR4 axis blocked metastasis of the mammary carcinoma cell line MDA-MB-231 to the lung, where CXCL12 is produced (Müller et al. 2001). In breast cancer patients, high levels of CXCR4 expression are associated with a relatively poor survival rate (Li et al. 2004). The tyrosine kinase receptor human epidermal growth factor receptor 2 (HER2)/neu, which is amplified or overexpressed in about 30 % of breast cancers, enhances the expression and function of CXCR4 by inhibiting CXCR4 degradation (Li et al. 2004). Similar to breast cancer cells, leukemia cells expressing CXCR4 home to bone marrow and access niches where stromal cells secrete CXCL12 (Burger et al. 1999; Burger and Kipps 2006). Also small-cell lung cancer cells express high levels of functional CXCR4 and home to bone marrow (Burger et al. 2003).

Further chemokine receptors have also been implicated in the dissemination of tumors into distant organs. For instance, CXCR1, CXCR2, and CXCR3 mediate metastasis of malignant melanoma cells (Gabellini et al. 2009; Singh et al. 2010; Amatschek et al. 2011).

The CX3CR1 receptor is implicated in the perineural invasion frequently observed in pancreatic adenocarcinoma (Marchesi et al. 2008) or in metastasis of prostate cancer cells into bone marrow (Shulby et al. 2004). Perhaps, the

interaction of this receptor with its transmembrane ligand CX3CL1 on endothelial cells allows tumor cells to transmigrate and extravasate into the bone stroma, as recently shown for breast cancer cells (Jamieson-Gladney et al. 2011).

Members of the CCR family are used by tumors to spread into secondary lymphoid organs and to skin, gut, or liver. CCR7 normally recruits activated dendritic cells to the lymph nodes, where its ligands CCL21 and CCL19 are expressed (Lira 2005). Analogous to this mechanism, many leukemia and lymphoma cells that express CCR7 are thus directed to these primary sites of metastasis (Alfonso-Pérez et al. 2006). Also, lymph node metastasis has been correlated with CCR7 expression in melanomas (Shields et al. 2007), gastric carcinoma, esophageal squamous cell carcinoma, non-small-cell lung cancer, colorectal carcinoma, and breast cancer (Raman et al. 2011). In addition, the CCL19/CCL21–CCR7 axis is also involved in further organ-selective metastasis: T-cell acute lymphoblastic leukemia frequently infiltrates the brain. In animal models it was shown that expression of CCR7 on leukemia cells and of CCL19 in the brain is essential for this organ-selective metastasis (Buonamici et al. 2009). Furthermore, in a murine model, CCR7 directs melanoma cells also to the skin (Fang et al. 2008).

Specifically in melanoma, the CCL27–CCR10 axis appears to have a fundamental role in supporting survival and metastasis in the skin (Ben-Baruch 2008; Richmond et al. 2009). CCL27 is constitutively expressed in the epidermis by keratinocytes, and melanoma cells express CCR10. Receptor expression is often associated with malignancy (Simonetti et al. 2006). Besides homing, CCR10 also allows melanoma cells to escape host immune antitumor killing mechanisms (Murakami et al. 2003).

CCR9 appears to be a fundamental homing receptor for melanoma metastasis to the small bowel, where the ligand CCL25 is highly expressed (Letsch et al. 2004). Namely, it has been shown that 88 of 102 small bowel melanoma metastases analyzed expressed CCR9 (Amersi et al. 2008).

Table 13.3 Examples of tumor metastases driven by chemokine ligand–receptor interactions

Chemokine receptor	Tumor type	Ligand	Metastasis site
CXCR4	Breast cancer	CXCL12	Liver (hepatocytes)
	SCLC		Lung, bone marrow (stromal cells)
CX3CR1	Pancreatic adenocarcinoma	CX3CL1	Perineural nets
	Prostate cancer		Bone
CCR7	Leukemias	CCL19/CCL21	Lymph nodes
	Diverse cancers		Lymph nodes
	T-ALL		Brain
	Melanoma		Skin
CCR10	Melanoma	CCL27	Skin (keratinocytes)
CCR9	Melanoma	CCL25	Small bowel

SCLC small-cell lung cancer, *T-ALL* T-cell acute lymphoblastic leukemia

Apart from the initial concept outlined above that tumor cells that express chemokine receptors are driven to sites of ligand production (Table 13.3), chemokine receptor–ligand interactions also apparently play a dominant role in the first steps of tumor metastasis: the dissemination from the parent tumor into lymphatic or blood vessels, from where the tumor cells spread to form organ metastases. It has been estimated that 80 % of solid cancers, especially breast cancer and melanoma, are distributed through the lymphatic system and 20 % are distributed through the bloodstream and by direct seeding (Leong and Witte 2011). To reach lymphatic and blood vessels, they are directed by CXCL12, CCL21, and perhaps other chemokines that are secreted by endothelial cells. Thus, cancer cells expressing the corresponding receptors may use the chemokine gradient to sense the direction to the vessels (Alitalo and Detmar 2012). Furthermore, cross talk between these chemokines and (lymph)angiogenesis factors such as VEGF-A, VEGF-C, and VEGF-D enhances this process (Kim et al. 2010).

Overall, chemokines apparently play a pivotal role in the metastasis of tumors and their organ-specific homing. However, in particular for organ-specific homing, besides chemokines , other growth, angiogenic, and chemotactic factors such as VEGFs, epidermal growth factors, transforming growth factor β, hepatocyte growth factor/scatter factor (Oxmann et al. 2008), proteases (Stark et al. 2007), and extracellular matrix components have to be taken into account (Steeg et al. 2011).

13.3 Conclusion and Perspectives

In the last decade compelling evidence has accumulated for the involvement of chemokines in cancer development, progression, and angiogenesis and site-selective metastasis of different types of cancers. In particular, the receptors CXCR4, CX3CR1, CCR4, CCR7, CCR9, and CCR10 appear to be of special importance. Small molecule receptor antagonists or neutralizing antibodies to receptors and ligands appear to be attractive tools to cure cancers, and especially to target cancer metastasis. However, despite growing cell biology and preclinical evidence, and some success in clinical studies with a few chemokine receptor antagonists, breakthroughs are still awaited (Wong and Korz 2008; Wu et al. 2009). Perhaps, the complexities of the system have to be understood in more detail, as far as mixtures of chemokines are involved, different chemokine receptors for the same ligand exist (e.g., clinical studies with CXCR4 antagonist did not take the novel receptor CXCR7 into account), and the interaction of chemokines with other growth factors has to be considered. Thus, further understanding of the chemokine–tumor network and the introduction of clinically applicable drugs remain challenges for the future.

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Chapter 14
Autophagy, Cell Death, and Cancer

Maryam Mehrpour, Ahmed Hamaï and Patrice Codogno

Abstract Macroautophagy or autophagy is a self-digesting mechanism in which the cellular contents are engulfed by autophagosomes and delivered to the lysosomes for degradation. Although it has been established that autophagy is an important protective mechanism for cells under stress from causes such as starvation by providing nutrients and removing protein aggregates and damaged mitochondria, the relationship between autophagy and cell death is complex. Autophagy can contribute to cell death via autophagic cell death or by being upstream of apoptosis. This complexity reflects the molecular overlap that exists between autophagy, apoptosis, and necrosis. The relationship between autophagy and cell death is only part of the role played by autophagy in cancer. Depending on the stage of tumor progression, autophagy can act as a tumor-suppressor mechanism or can help tumor cells cope with stressful situations generated by the tumoral environment or cancer treatment.

Keywords Apoptosis • Macroautophagy • Necrosis • Tumor initiation • Tumor progression

14.1 Introduction

The term "autophagy" is used to describe three types of mechanism: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). Macroautophagy and microautophagy are conserved in all eukaryotic cells, whereas CMA is restricted to mammalian cells. Microautophagy is the direct engulfment of cytoplasmic material by the lysosomal membrane, whereas in CMA the lysosomes import KFERQ-containing proteins via their interaction with the chaperone hsc70 and the lysosomal membrane protein LAMP-2a (Orenstein and Cuervo 2010). For further information about the

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molecular aspects and biological role of CMA, readers should consult a recent review (Kaushik and Cuervo 2012). Macroautophagy (referred to in the following simply as autophagy) is a homeostatic and conserved eukaryotic process that ensures the digestion of long-lived cytoplasmic components via the lysosomal pathway (Yang and Klionsky 2010a). Autophagy is a multistep process that starts with the formation of a double-membraned vacuole, known as an autophagosome, which engulfs damaged proteins and organelles. This vacuole then fuses with the endocytic compartment and ultimately with lysosomes, resulting in the degradation of the sequestered material (Kuma and Mizushima 2010). Although another cellular degradation component, known as the proteasome, is also able to degrade proteins, only autophagy can degrade long-lived macromolecules and entire organelles, thus ensuring the turnover of cellular materials and the recycling of intracellular constituents (such as amino acids, fatty acids, and nucleotides) (Mizushima 2011). Autophagy has been described as a "self-eating" process, and in most cells it ticks over at a basal rate, acting as a cytoplasmic quality-control mechanism and getting rid of protein aggregates and damaged organelles in order to maintain tissue homeostasis (Mizushima and Komatsu 2011). In response to starvation, nutrient deprivation, growth factor depletion, or hypoxia, autophagy becomes an important stress-induced process that maintains metabolism and ATP levels, and helps the cell to survive (Singh and Cuervo 2011). The physiological importance of autophagy in maintaining energy homeostasis in various tissues and, indeed, enabling the organism to survive has been demonstrated in vivo in newborn mice following the sudden withdrawal of the supply of maternal nutrients via the placenta (Kuma and Mizushima 2010). Other studies have highlighted the importance of autophagy in various aspects of both innate and adaptive immune responses, including pathogen clearance, the production of type I interferon, antigen presentation, and lymphocyte development, as well as the downregulation of cytokine signaling and inflammation (Virgin and Levine 2009; Levine et al. 2011; Deretic 2012). In this chapter, after briefly describing the physiological and pathological regulation and roles of autophagy, we will focus on the role of autophagy in cell death and cancer.

14.2 Regulation of Autophagy

Autophagy is orchestrated by the coordinated action of evolutionarily conserved autophagy-related (Atg) proteins with their partners to form the autophagosome, which is derived from the preautophagosomal structure to which Atg proteins are hierarchically recruited (as summarized in Fig. 14.1). The ULK1 complex (a complex composed of ULK1, the mammalian orthologue of yeast Atg1, and FIP200, the mammalian functional homologue of yeast Atg13, Atg17, and Atg101), and the phosphatidylinositol 3-kinase (PI3K) complex (a complex composed of Beclin-1, the mammalian orthologue of yeast Atg6, Atg14, Vps34, hVps15, and AMBRA1) congregate at the preautophagosomal structure to initiate autophagy (Mizushima et al. 2011). The production of phosphatidylinositol 3-phosphate by hVps34 recruits the phosphatidylinositol 3-phosphate binding proteins WIPI-1/2 (Atg18) and DFPC1, which

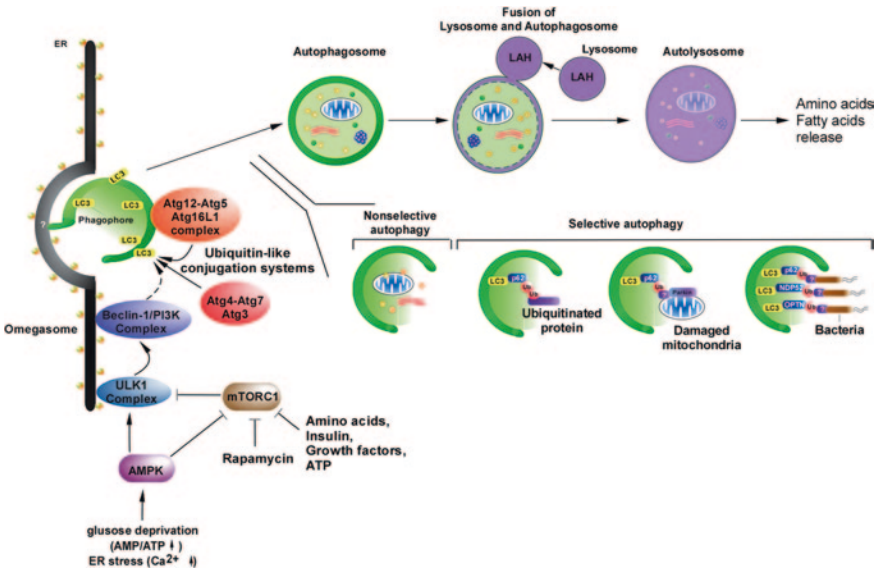

Fig. 14.1 The autophagy pathway. Autophagy is orchestrated by the coordinated action of autophagy-related (*Atg*) proteins to form the autophagosome from the preautophagosomal structure, to which Atg proteins are hierarchically recruited to form the isolation membrane of the phagophore. Through a process of maturation and fusion, these membrane-bound structures become autolysosomes, degrading their contents and releasing amino acids, fatty acids, nucleotides, and other molecules required to maintain cell metabolism. The selective autophagy mediated by autophagy receptors, such as p62 here, ensures that ubiquitinated structures, including protein aggregates, damaged mitochondria, and microbes, are eliminated. Autophagy is principally regulated by the sensors of nutrient and energy levels mammalian target of rapamycin complex 1 (*mTORC1*) and AMP-activated protein kinase (*AMPK*). *ER* endoplasmic reticulum, *LAH* lysosomal acid hydrolases

promote the elongation and closure of the membrane, thus accommodating and isolating the two ubiquitin-like conjugation systems (Atg12–Atg5/Atg16L1 and phosphatidylethanolamine-conjugated LC3/GABARAP, the mammalian orthologue of yeast Atg8). Thus, autophagy, which is a multistep process characterized by the hierarchical assembly of proteins, must itself be tightly regulated in order to respond appropriately to numerous different types of stimuli (Mizushima et al. 2011). Several signaling pathways, known as cellular sensor pathways, have been identified and shown to be able to respond to these different types of stimuli (growth factors, energy status, oxygen, glucose, amino acids, and etc.). The first sensor to be identified was mammalian target of rapamycin (mTOR) complex 1 (mTORC1), which induces autophagy when it has been inhibited by rapamycin, and is conserved from yeast to mammals. The activity of mammalian target of rapamycin (mTOR) is regulated by the class I PI3K–Akt pathway (Meijer and Codogno 2009). Under starvation conditions or in response to rapamycin treatment, mTORC1, which controls the kinase activity of ULK1, dissociates from the ULK1 complex, resulting in the activation of ULK1 and in the initiation of autophagy. Other sensor pathways have since been identified, including

AMP-activated protein kinase, which is a sensor of nutrient and energy levels, and which activates autophagy via at least two mechanisms—the inhibition of mTOR and direct activation of ULK1 (Meijer and Codogno 2009).

Once formed, the autophagosomes can merge with endocytic compartments (e.g., early and late endosomes, multivesicular bodies) before fusing with the lysosomal compartment (Stromhaug and Seglen 1993; Liou et al. 1997; Tooze and Razi 2009). The term "amphisome", from the Greek roots *amphi* ("both") and *soma* ("body"), was coined by Per O. Seglen to describe the vacuole resulting from the fusion of the autophagosome and the endosome (Berg et al. 1998). The late stage of autophagy depends on molecules that regulate maturation of autophagosomes, including their fusion with endosomes and lysosomes, the acidification of the autophagic compartments, and the recycling of metabolites from the lysosomal compartment. These steps are fundamental for the flux of material through the autophagic pathway (defined here as spanning from the cargo-sequestration step through to lysosomal degradation). Any blockade of the maturation or fusion of the autophagosome with the lysosomal compartment, or impairment of lysosomal function or biogenesis, results in an accumulation of autophagosomes that inevitably slows down or disrupts the autophagic flux (Boya et al. 2005; Rubinsztein et al. 2009).

Autophagy is a bulk process, but in many cases the process can be highly selective for specific cellular structures, including peroxisomes (pexophagy), mitochondria (mitophagy), endoplasmic reticulum (ER) (reticulophagy), ribosomes (ribophagy), mid-body, micronuclei and parts of the nucleus (nucleophagy), lipid droplets (lipophagy), aggregation-prone proteins (aggrephagy), secretory granules (zymophagy), and microorganisms (xenophagy) (for recent a review, see Reggiori et al. 2012). The paradigm for selective autophagy is the recognition of the cargo by an autophagy receptor (SQSTM1/p62, NBR1, NDP52, optineurin) that connects the cargo to the autophagy machinery (Fig. 14.1). For example, the autophagy receptor p62 recognizes aggregation-prone proteins via its ubiquitin-associated domain and the protein LC3 via its LC3-interacting region motif. In many autophagy receptors, phosphorylation consensus sites are present in the vicinity of the LC3-interacting region motif. Phosphorylation of these sites has been shown to increase the affinity of the autophagy receptor for LC3. Defective selective autophagy is accompanied by accumulation of cargo and reduced turnover of autophagy receptors. Both of these can disrupt tissue homeostasis and lead to disease. For example, accumulation of the autophagy receptor p62 leads to activation of nuclear factor κB (NF-κB) and the transcription factor NRF2, two events linked with tumorigenesis and the accumulation of bacteria resulting in chronic infection (Mizushima and Komatsu 2011).

14.3 Autophagy in Physiology and Pathology

Autophagy is induced in cells as a result of many different stressful situations, such as starvation, hypoxia, and infection (Fig. 14.2), and its fine regulation is essential to maintain cellular and tissue homeostasis (Kroemer et al. 2010). The stimulation of autophagy during periods of starvation is an evolutionarily

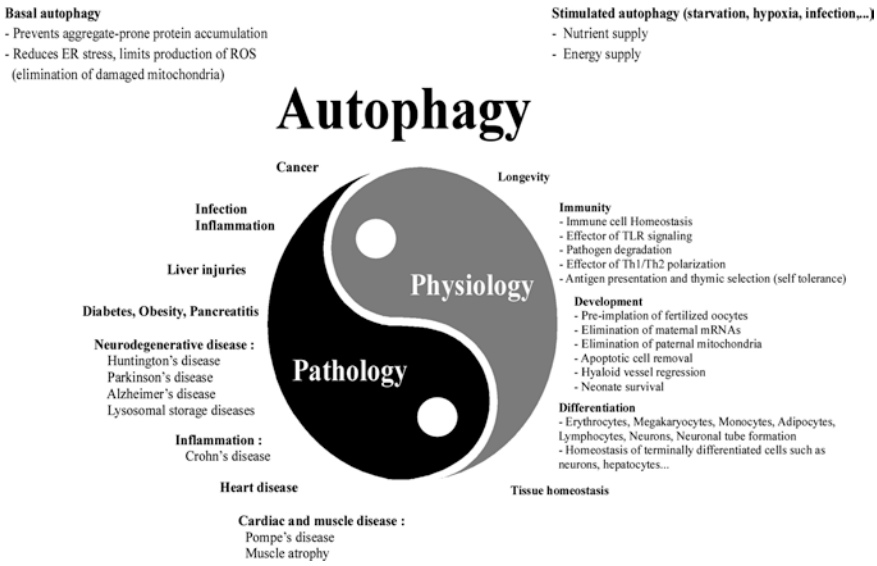

Fig. 14.2 The physiological and physiopathological roles of autophagy. Autophagy has many essential functions at both the cell and the tissue level. Basal autophagy is essential to prevent the accumulation of damaged proteins and organelles; it reduces ER stress and limits the production of reactive oxygen species (*ROS*). On the other hand, autophagy stimulated by factors such as starvation, hypoxia, and infection is important to provide the nutrients and building blocks and supply energy during periods of cellular stress. The "yin"—autophagy—is essential during the development and differentiation of many cell types and for maintaining tissue homeostasis. It also plays a key role in immunity, which involves thymic selection, antigen presentation, and pathogen degradation. Autophagy is also important for longevity as it maintains cellular homeostasis during aging. The "yang" aspect: it is not surprising that dysregulation of autophagy has profound implications and has been found involved in the pathogenesis of many disorders. For example, defects in autophagy have been associated with cancer and with numerous neurodegenerative diseases, including Huntington's, Alzheimer's, and Parkinson's diseases, lysosomal storage diseases, and have also been reported in liver and muscle disorders. Others pathological situations, such as diabetes and obesity, and inflammatory disorders such as Crohn's disease, have also been linked to defective autophagy. *mRNAs* messenger RNAs, *TLR* Toll-like receptor

conserved response to stress in eukaryotes (Yang and Klionsky 2010b). Under starvation conditions, the degradation of proteins and lipids allows the cell to adapt its metabolism and meet its energy needs. The stimulation of autophagy plays a major role at birth, thus maintaining energy levels in various tissues after the maternal supply of nutrients via the placenta ceases (Kuma et al. 2004). Furthermore, the pharmacological and genetic downregulation of autophagy induces rapid cell death following starvation in cells (Boya et al. 2005).

Autophagy is also essential during development and differentiation (Fig. 14.2). The preimplantation period after oocyte fertilization is dependent on the autophagic degradation of components of the oocyte cytoplasm, e.g., elimination of maternal messenger RNAs (Tsukamoto et al. 2008) and paternal mitochondria (Al Rawi et al. 2011; Sato and Sato 2011). Autophagy is also implicated in the elimination of apoptotic bodies generated during naturally occurring cell death

associated with embryonic development (Mellén et al. 2008, 2009). Autophagy-induced remodeling of the cytoplasm is involved in the differentiation of erythrocytes, lymphocytes, megakaryocytes, monocytes, and adipocytes (Ravikumar et al. 2010). Autophagy is also necessary for the differentiation of adult embryonic stem cells into neurons (Vazquez et al. 2012). Moreover, autophagy is crucial for the homeostasis of immune cells, and contributes to the regulation of self-tolerance (Nedjic et al. 2009). Autophagy is also essential to maintain hematopoietic stem cells (Mortensen et al. 2011). Induction of autophagy during calorie restriction may contribute to the observed extension of lifespan in rats. Recent data have shown that the induction of autophagy increases longevity in a wide variety of species (Rubinsztein et al. 2011). This antiaging effect probably depends, at least in part, on the quality-control function of autophagy, which limits the accumulation of aggregation-prone protein and damaged mitochondria.

As described above, autophagy is essential to eliminate many harmful components from cells, such as protein aggregates, damaged organelles, and intracellular pathogens. It is therefore not surprising that disruption of this process has serious consequences, and is implicated in many diseases, including Huntington's, Alzheimer's and Parkinson's diseases, all of which are characterized by the accumulation of protein aggregates in the brain (Cheng et al. 2010) and in other tissues, such as muscle (Ravikumar et al. 2010), and in liver fibrosis (Hidvegi et al. 2010.). In the heart, basal autophagy is necessary to maintain cellular homeostasis and is upregulated in response to stress in hypertensive heart disease, heart failure, cardiac hypertrophy, and ischemia–reperfusion (Nakai et al. 2007). In the pancreas, autophagy is required to maintain the architecture and function of pancreatic β cells (Ebato et al. 2008). Defective hepatic autophagy probably contributes to insulin resistance and to predisposition to type 2 diabetes and obesity (Yang et al. 2010). Decreased hepatic autophagy is observed in both genetic and dietary mouse models of obesity and insulin resistance (Yang et al. 2010). This impacts on ER function, including the response to stress. The restoration of ATG7 expression limits the obesity-dependent ER stress and rescues insulin resistance and glucose tolerance. However, autophagy has the opposite effect in the white adipose tissue, where its inhibition reduces the white adipose mass and enhances insulin sensitivity (Singh et al. 2009a; Zhang et al. 2009). The adipose-specific deletion of *Atg7* also promotes the oxidation of free fatty acids by increasing the proportion of brown adipocytes, leading to a leaner body (Singh et al. 2009a). The effects of the pharmacological manipulation of autophagy in obese patients thus remain uncertain, unless liver autophagy can be specifically targeted (Codogno and Meijer 2010). Autophagy is involved not only in regulating metabolism in the peripheral tissues, but also in regulating food intake via the brain (Kaushik et al. 2011; Meng and Cai 2011), although its role in this process remains to be clearly demonstrated.

Given its role in the elimination of intracellular pathogens (bacteria, viruses, and parasites), autophagy also contributes to innate immunity (Deretic 2011). Recently, polymorphism of the genes that encode Atg16L1 and IRGM, two autophagy genes essential for the elimination of intracellular pathogens, has been associated with Crohn's disease, a chronic inflammatory bowel disease (Virgin and Levine 2009).

Amino acids produced by autophagy in the muscles and liver can be used for gluconeogenesis in the liver (Rabinowitz and White 2010), and can contribute to the production of ATP by entering the tricarboxylic acid cycle. Degradation of liver lipid droplets by autophagy, via lipophagy, contributes to the generation of free fatty acids that are oxidized in the mitochondria (Singh et al. 2009a). Moreover, hepatocyte-specific *Atg7*-knockout mice exhibit elevated levels of hepatic lipids (Singh et al. 2009b).

14.4 Autophagy and Cell Death

Programmed cell death is one of the homeostatic processes that are essential for the elimination of unwanted or harmful cells during the development and maintenance of multicellular organisms (Elmore 2007). Dysregulation of cell death leads to a wide range of disorders including aberrant embryogenesis, neurodegenerative disease, and cancer (Lockshin and Zakeri 2001; Strasser et al. 2011). The physiological and pathological importance of cell death has prompted great interest over the last 20 years. Nonetheless, apparently simple questions concerning the very definition of cell death and the classification of cell death modalities in stereotyped patterns have not yet been answered (Table 14.1). There are at least three morphologically distinct types of cell death, and these have been designated "apoptosis," "autophagic cell death" (ACD), and "necrosis," respectively (Edinger and Thompson 2004; Amaravadi and Thompson 2007; Galluzzi et al. 2011b). Apoptosis is morphologically defined by cellular condensation, membrane blebbing, nuclear shrinkage and fragmentation, and the formation of apoptotic bodies (Kerr et al. 1972). For a long time, necrosis was regarded as an uncontrolled form of cell death, but accumulating evidence indicates that necrotic cell death is in fact conducted by complex signal transduction pathways regulating execution mechanisms. Necroptosis (a regulated form of necrosis) is morphologically defined by cytoplasmic swelling, early permeabilization, and breakdown of the plasma membrane (Golstein and Kroemer 2007; Christofferson and Yuan 2010). By definition, ACD is morphologically characterized by the massive sequestration of portions of the cytoplasm within the autophagosomes, giving the cell a characteristic vacuolated aspect (Shintani and Klionsky 2004; Tsujimoto and Shimizu 2005; Mehrpour et al. 2010). However, the classification of cell death should never be based on morphological criteria alone, but should also take into account the immunological or biochemical criteria data (or any other data available) in order to delineate each distinct experimental model correctly (Galluzzi et al. 2011a).

14.4.1 Autophagy and ACD

It seems that autophagy may, in fact, be a key regulator of both apoptosis and necrosis. Autophagy provides protection against cell death by its ability to counteract the

Table 14.1 Cell death modalities

	Apoptosis	Autophagy	Necrosis (necroptosis)
Morphology	Cell shrinkage Membrane blebbing Reduction of cellular and nuclear volume (pyknosis) Nuclear fragmentation	Massive sequestration of portions of the cytoplasm within autophagosomes Lack of chromatin condensation	Cytoplasmic swelling Membrane breakdown Cell contents released
Key biochemical features	Activation of proapoptotic Bcl-2 family proteins (e.g., Bax, Bak, Bid)	Dissociation of Beclin-1 from Bcl-2/Bcl-xL	Specific PARP1 cleavage pattern
	Activation of caspases	Dependency on Atg gene products and mTOR suppression	HMGB1 release
	Accumulation of ssDNA Oligonucleosomal DNA fragmentation	Conversion of LC3-I to LC3-II Degradation of p62/SQSTM1	Drop in ATP levels
	Phosphatidylserine exposure	Phosphatidylserine exposure	RIP1 phosphorylation and ubiquitination
Key events at the organelle level	Mitochondrial outer membrane permeabilization Dissipation of mitochondrial transmembrane potential	Autophagosome and lysosome fusion	Lysosome membrane permeabilization
Key physiological features	Overgeneration of ROS Suppression of inflammation	Overgeneration of ROS Suppression of inflammation	Overgeneration of ROS Inflammation
Chemical inhibitors	ZVAD, QVD	3-Methyladenine, wortmannnin, chloroquine	Nec-1

Atg autophagy-related, *Bcl-2* B cell lymphoma 2, *HMGB1* high mobility group box 1, *mTOR* mammalian target of rapamycin, *PARP1* poly(ADP-ribose) polymerase 1, *RIP1* receptor-interacting protein 1, *ROS* reactive oxygen species, *ssDNA* single-stranded DNA

cell damage produced by toxic substances and energy depletion. However, autophagy can act at several different stages in the cell death cascade. Although the expression "autophagic cell death" (ACD) suggests that cell death is executed by autophagy, recent data from Shen et al. 2011, who used high-throughput chemical screens, failed to demonstrate that any of the compounds tested actually killed cells via autophagy. The results of such studies could be influenced by a possible role of ATG genes in other functions involved in cell death unrelated to autophagy. One must also be cautious in these cases because ACD is operative in midgut cell death in *Drosophila melanogaster* (Denton et al. 2009) and during differentiation in *Dictyostelium discoideum* (Giusti et al. 2009). In the latter situation, inhibition of autophagy reveals necrotic cell death. In mammals, ACD reportedly occurs in a model of neurodegeneration (Koike et al. 2008); however, no in vivo data are available from mammalian physiological settings (Debnath et al. 2005) (Kroemer and Levine 2008). Some scientists argue that rapidly dividing mammalian cells, such as cancer cells, are not appropriate to find ACD (Clarke and Puyal 2012). Mice with defective autophagy could provide useful models of these situations, as suggested by Kimmelman (2011) (Table 14.1).

Autophagy may protect cells, including detached cells harboring antiapoptotic lesions, against anoikis (cell death through the loss of the extracellular matrix interaction) (Fung et al. 2008). Despite a lack of nutrients (Boya et al. 2005) or if the uptake of extracellular nutrients is impaired because of a lack of growth factors (Lum et al. 2005), autophagy can preserve cells from apoptosis by maintaining an intracellular supply of substrates. If autophagy is inhibited, CMA has been shown to protect cells against some death-inducing stimuli such as reactive oxygen species (ROS) and UV light (Wang et al. 2008). The accumulation of ROS produced in response to many cellular stresses such as starvation and cancer drugs can directly inactivate autophagy by inactivating cysteine protease Atg4. This leads to the accumulation of the LC3-phosphoethanolamine precursor, which is required for autophagosome formation (Scherz-Shouval et al. 2007).

14.4.2 Apoptosis

Apoptosis can be triggered either at the cell surface (the extrinsic pathway) or in the mitochondria (the intrinsic pathway) (Hengartner 2000; Fuchs and Steller 2011; Kantari and Walczak 2011). Extracellular signals may include toxins, hormones, growth factors, nitric oxide, and cytokines that must either cross the plasma membrane or be transduced in order to elicit a response. One of the most investigated extrinsic pathways, that of the death receptors that are members of the tumor necrosis factor (TNF) receptor superfamily, is activated by binding of the natural ligands of these receptors [TNFα, FasL, TNF-related apoptosis-inducing ligand (TRAIL)] (Walczak and Krammer 2000; Ashkenazi 2002; Muppidi et al. 2004; Gonzalvez and Ashkenazi 2010). After activation, the receptor death domains attract the intracellular adaptor protein Fas-associated death domain protein (FADD; also known as MORT1), which, in turn, recruits the inactive proforms of certain members of the caspase

protease family. The caspases that are recruited to this death-inducing signaling complex (DISC)—caspase 8 and caspase 10—function as "initiator" caspases (Chen et al. 2002). At the DISC, procaspase 8 and procaspase 10 are cleaved and yield active initiator caspases. The DISC may also contain one or both isoforms of cellular FLICE-inhibitory protein (c-FLIP), which, depending on the context, may either inhibit or enhance caspase 8 activation (Thome and Tschopp 2001; Wilson et al. 2009).

In some cells, known as type I cells, the amount of active caspase 8 formed at the DISC is sufficient to initiate apoptosis directly, but in type II cells (hepatocytes and pancreatic β cells), the amount is too small and mitochondria are used as "amplifiers" of the apoptotic signal (Salvesen and Ashkenazi 2011). In response to triggering the death receptor, the activation of caspase 8 may result in cleavage of the BH3-only protein Bid (Ozoren and El-Deiry 2002), which in turn translocates to mitochondria to stimulate mitochondrial outer membrane permeabilization (MOMP), thereby initiating a mitochondrial amplification loop (Kantari and Walczak 2011).

The intrinsic pathway involves MOMP, including the dissipation of mitochondrial transmembrane potential and the release of apoptogenic factors, such as cytochrome *c*, AIF and Smac/DIABLO, from the mitochondrial intermembrane space into the cytosol (Green and Reed 1998; Kroemer 1998; Wang 2001). Cell-intrinsic stimuli control MOMP through the proteins of the multidomain B cell lymphoma 2 (Bcl-2) family (Lindsten et al. 2000). Members of this family are evolutionarily conserved and can be divided into three subgroups according to their function or structural similarity within four Bcl-2-homology domains (designated BH1–BH4) (Cory and Adams 2002; Petros et al. 2004; Youle and Strasser 2008).

Together with the cytoplasmic adaptor protein Apaf1 and dATP, cytochrome *c* drives the assembly of the apoptosome, another caspase-activating multiprotein complex, with activation of the apical caspase, caspase 9 (Li et al. 1997). Caspase 9 activates the effector caspases 3, 6, and 7, which cleave several hundred cellular proteins, resulting in the characteristic biochemical and morphological features associated with apoptosis, including plasma membrane "blebbing," cell shrinkage, chromatin condensation, and internucleosomal DNA fragmentation. In vivo, apoptotic cells maintain their plasma membrane integrity, and are rapidly phagocytosed in the absence of an inflammatory response (Taylor et al. 2008). However, in vitro at late stages of incubation, apoptotic cells may undergo secondary necrosis when the plasma membrane loses its integrity and becomes more permeable.

14.4.3 Necrosis

The initiation of programmed necrosis, known as "necroptosis," by death receptors requires the kinase activity of receptor-interacting protein 1 and receptor-interacting protein 3, and its execution involves the active disintegration of mitochondrial, lysosomal, and plasma membranes. When cells deficient in Bax and Bak are treated with alkylating chemotherapy, an immediate metabolic crisis develops, which is characterized by NAD^+ depletion followed by ATP depletion. A key mediator in

this process is poly(ADP-ribose) polymerase, a nuclear protein which when activated by DNA damage rapidly depletes the cell of NAD^+, the essential cofactor for aerobic glycolysis. After signaling- or damage-induced lesions, necrosis can include signs of controlled processes such as mitochondrial dysfunction, enhanced calcium generation (Xu et al. 2001), ROS (Lin et al. 2004), ATP depletion, proteolysis by calpains (Syntichaki et al. 2002) and cathepsins, and early plasma membrane rupture (Amaravadi and Thompson 2007). High mobility group box 1 (HMGB1), a highly conserved nuclear protein, acts as a chromatin-binding factor that bends DNA and promotes access to transcriptional protein complexes (Harris et al. 2012). Under stressful conditions, HMGB1 is released and promotes inflammation. HMGB1 is passively released by necrotic but not by apoptotic death of normal cells, and is actively secreted by a variety of activated immune and nonimmune cells. In addition to its nuclear role, HMGB1 also acts as an extracellular signaling molecule during inflammation, cell differentiation, cell migration, wound healing, and tumor progression (Lotze and Tracey 2005; Tang et al. 2010b). Space does not allow us to discuss this in detail, and readers interested in the regulation of necroptosis by signaling pathways and in the role of necroptosis are referred to recent reviews of these topics (Vandenabeele et al. 2010; Galluzzi et al. 2011a).

14.4.4 Cross Talk Between Autophagy, Apoptosis, and Necrosis

Accumulating evidence suggests the existence of several molecular connections among autophagy, apoptosis, and necrosis. A excellent review has recently discussed the various aspects of this cross talk (Gordy and He 2012). In response to specific perturbations, the same input signal can cause cells to change from one cell death manifestation to another, with a mixed type of cell death also being observed in some cases. In cells undergoing persistent autophagy, hallmarks of apoptosis, such as caspase activation, necrotic cell death, organelle swelling, and plasma membrane rupture, are often observed. In contrast, autophagy protects nutrient-deprived or growth-factor-withdrawn cells, and allows them to survive by inhibiting apoptosis.

In the presence of caspase inhibitors, autophagy also protects the cells from caspase-independent death. Depending on the cellular setting, the same proteins can regulate both autophagic and apoptotic processes. For example, protein p53, a potent inducer of apoptosis, also activates autophagy via its target gene, damage-regulated modulator of autophagy (DRAM)) (Crighton et al. 2006).

14.4.4.1 Autophagy and Extrinsic Apoptosis

In addition to well-defined roles in extrinsic apoptosis (see Sect. 14.4.2), studies of cells with inactivated caspase 8 or defective FADD–procaspase 8 interactions have suggested that the key components of the DISC also modulate autophagy

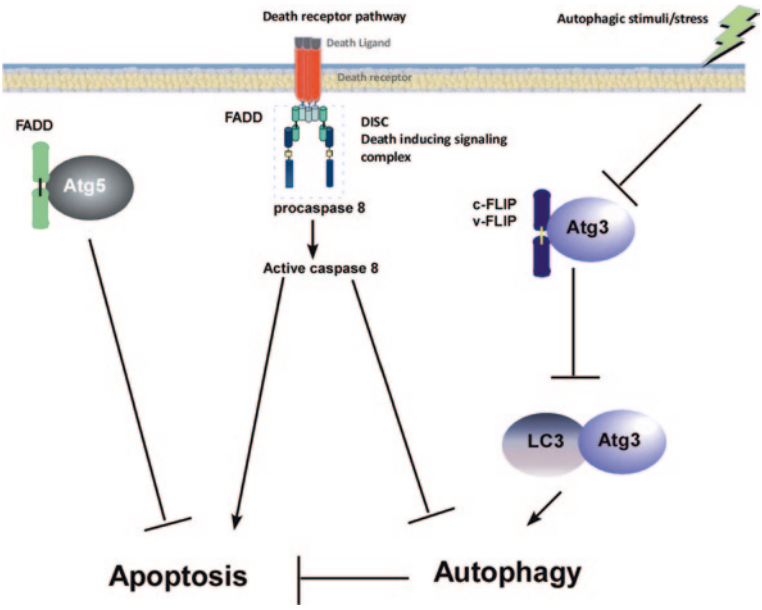

Fig. 14.3 The extrinsic apoptotic and autophagic pathways. Death receptors are activated by their binding to their natural ligands. This results in the recruitment of Fas-associated death domain protein (*FADD*) (Walczak and Krammer 2000; Ashkenazi 2002; Muppidi et al. 2004; Gonzalvez and Ashkenazi 2010), which, in turn, recruits the inactive proforms of caspase 8 and some other members of the caspase protease family. Procaspase 8 cleavage results in the release of active caspase 8, which mediates apoptosis and inhibits autophagy by an unknown mechanism. The caspase 8 inhibitory proteins cellular FLICE-inhibitory protein (*c-FLIP*) and viral FLICE-inhibitory protein (*v-FLIP*) interact with Atg3, thereby preventing the interaction between Atg3 and LC3, and inhibiting autophagy to the death-inducing signaling complex (*DISC*). Under starvation conditions, FLIP–Atg3 interaction is inhibited, allowing autophagy to proceed. Similarly, FADD interacts directly with Atg5; however, this interaction appears to inhibit apoptosis rather than autophagy

(Fig. 14.3). Inhibition of caspase 8 or its deficiency results in excessive autophagy in fibroblasts, macrophages, and T cells (Yu et al. 2004; Bell et al. 2008). Similarly, expression of an FADD mutant that contains the death domain (FADD-DD), which allows it to be recruited to the DISC, but not to the death effector domain, which mediates its interaction with procaspase 8 and c-FLIP, inhibits death-receptor-induced apoptosis and induces excess levels of autophagy in epithelial cells and T cells (Thorburn et al. 2005; Bell et al. 2008).

Moreover, Atg5 has been shown to interact with FADD through its death domain; however, FADD deficiency does not affect autophagosome formation (Pyo et al. 2005). Pyo et al. (2005) showed that the loss of FADD rescued the increased caspase-dependent cell death observed in response to the overexpression of Atg5, suggesting that the Atg5–FADD interaction may serve to regulate apoptosis rather than autophagy.

Further evidence for cross talk between the extrinsic apoptotic pathway and autophagy has been provided by Jung's laboratory. cFLIP and viral FLICE-inhibitory protein (v-FLIP) from Kaposi-sarcoma-associated herpesvirus, herpesvirus saimiri, and molluscum contagiosum virus all protect cells from apoptosis mediated by death receptors by preventing procaspase 8 cleavage.

Some authors have shown that both c-FLIP and v-FLIP interact with Atg3, thus preventing its interaction with LC3 and the induction of autophagy (Lee et al. 2009). This antiautophagic function is independent of the antiapoptotic and cellular activation functions of FLIP, as mutant v-FLIP constructs that are unable to bind FADD or TNF receptor associated factor 2 or to activate NF-κB can still inhibit autophagy.

LC3–Atg3 interaction was inhibited by proautophagic stimuli, such as starvation and rapamycin treatment, suggesting that a fine balance between c-FLIP and Atg3 expression levels may mediate cell fate decisions. In all these studies, the excess autophagy observed in cells with inhibited extrinsic apoptotic pathways was associated with increased cell death (Yu et al. 2004; Pyo et al. 2005; Bell et al. 2008; Lee et al. 2009); however, there is little evidence to suggest that autophagy was the direct cause of death of these cells.

Although evidence that Atg5 is required for both autophagosome formation and cell death in response to interferon-γ stimulation is convincing, the finding that truncation of Atg5 can directly induce apoptosis raises a possible alternative explanation: the Atg5-mediated promotion of cell death may be independent of its autophagic function (Pyo et al. 2005; Yousefi et al. 2006). Moreover, although the components of the DISC cooperate to regulate apoptosis, these findings also suggest that these proteins may have distinct roles in the regulation of autophagy. The enhanced autophagy observed in cells expressing FADD-DD appears to be due solely to the inability of FADD-DD to bind procaspase 8, as v-FLIP inhibits autophagy regardless of its ability to bind FADD. Thus, it appears that procaspase 8 must be recruited to the DISC to inhibit autophagy; considering the role of downstream caspases in the cleavage of Atg, caspase 8 activation may also be required. In contrast, c-FLIP (and v-FLIP) appears to play a DISC-independent role in the inhibition of autophagy; however, additional imaging studies will be necessary to determine the localization of c-FLIP during its interaction with Atg3.

14.4.4.2 Cleavage of Atg Proteins by Calpain and Caspase

The effector proteases of apoptosis (caspases) have been shown to inhibit autophagy through the cleavage of Atg proteins. The first direct evidence that apoptosis-associated proteases regulate the balance between apoptosis and autophagy was the observation that calpain 1 and calpain 2 mediated cleavage of Atg5 in human neutrophils undergoing spontaneous apoptosis (Yousefi et al. 2006). Truncated Atg5 (N-terminal cleavage product) was detected in multiple cell types, and was found to translocate to the mitochondria, where it associated with Bcl-xL to induce release of cytochrome *c* (Yousefi et al. 2006) (Fig. 14.4). Overexpression of this truncated Atg5

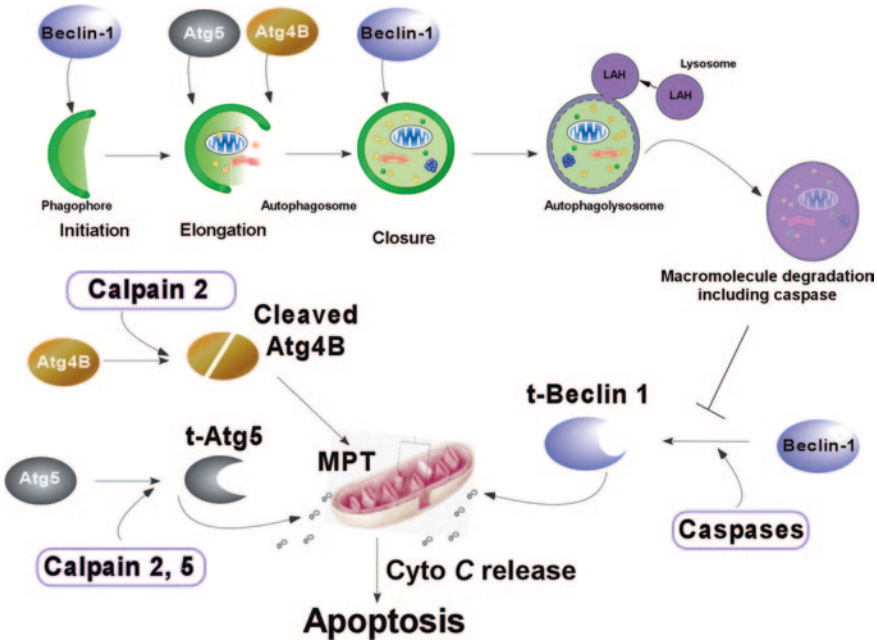

Fig. 14.4 Reciprocal negative regulation of autophagy and apoptosis. The autophagic functions of Beclin-1 and Atg5 proteins are downregulated by apoptotic signaling. Beclin-1 is cleaved by caspases (3, 6, 9, and 10), and Atg4B and Atg5 are cleaved by calpain 2 or by calpain 1 and calpain 2, respectively. These cleavage events serve not only to inhibit autophagy, but also to enhance apoptosis, as truncated forms of Beclin-1 (*t-Beclin-1*) and Atg5 (*t-Atg5*) are both translocated to the mitochondria, where they induce release of cytochrome *c* and cleaved Atg4B is associated with the onset of the mitochondrial permeability transition (*MPT*) and cell death. Full-length Beclin-1 and Atg5 oppose this process by mediating the autophagic degradation of active caspase 8. *LAH*, lysosomal acid hydrolases

was sufficient to cause nuclear condensation, demonstrating the ability of cleaved Atg5 to induce apoptosis directly. In contrast, the N-terminal cleavage product was not sufficient to induce autophagy. Importantly, these findings explain the ability of Atg5 overexpression to sensitize tumor cells to apoptosis-inducing anticancer agents such as ceramide, etoposide, doxorubicin, and CD95. As overexpression of LC3 had no effect on apoptosis, the enhanced sensitivity to apoptosis observed in Atg5-overexpressing cells cannot be due to enhanced autophagy, but must be solely attributable to the proapoptotic function of the truncated Atg5 generated by calpain-mediated cleavage. Similarly to the action of calpains, following either intrinsic or extrinsic apoptotic stimulation, caspases also cleaved Atg molecules, including Beclin-1 (Cho et al. 2009; Luo and Rubinsztein 2010; Norman et al. 2010; Wirawan et al. 2010; Zhu et al. 2010). Truncated Beclin-1 (a C-terminal cleavage product) translocated to the mitochondria to induce the release of cytochrome *c*. Only full-length Beclin-1 can induce autophagy, as in cells ectopically expressing truncated Beclin–1 both growth factor withdrawal and staurosporine treatment resulted instead

in enhanced apoptosis. Moreover, cleavage of Beclin-1 disrupts its interaction with Bcl-2; the C-terminal Beclin-1 cleavage product maintains a weak interaction with Bcl-2, whereas the interaction between Bcl-2 and the N-terminal cleavage product is completely lost (Zhu et al. 2010). Expression of the N-terminal cleavage product, but not that of the C-terminal cleavage product, partially restored autophagosome formation in Beclin-1 cells expressing short hairpin RNA, suggesting that caspase-mediated disruption of the Beclin-1–Bcl-2 complex may serve to promote autophagy (Zhu et al. 2010). These recent studies suggest that the balance between truncated and full-length Beclin-1 is reflected in the balance between apoptotic and autophagic cell fates (Cho et al. 2009; Wirawan et al. 2010; Zhu et al. 2010). Similarly, caspase 3 mediated cleavage of the autophagy-related endopeptidase Atg4D was shown to cleave and delipidate GABARAP-L1, resulting in a decrease in the number of GABARAP-L1 puncta, but not in that of LC3 puncta (Betin and Lane 2009). In a subset of staurosporine-treated cells, Atg4D was shown to localize to the mitochondria before cell death regardless of its cleavage status. However, the implications of the mitochondrial localization of Atg4D remain unclear, as apoptosis occurred in staurosporine-treated cells in which no Atg4D was observed in the mitochondria. Calpain 2 activation contributes to Atg4B depletion in old hepatocytes and firmly support the key role of Atg4B in ischemia and reperfusion injury (Wang et al. 2011). Taken together, these findings suggest a common process whereby Atg proteins are cleaved by apoptosis-related proteases, resulting in a shift in the balance between autophagy and apoptosis. The ability of at least some of these cleaved proteins to directly mediate apoptotic functions, such as release of cytochrome *c*, demonstrates the complexity of the interactions underlying this balance.

14.4.4.3 Beclin-1–Bcl-2 Family Interaction

In addition to the role of the balance between truncated and full-length Beclin-1 in regulating autophagy, direct interactions between full-length Beclin-1 and Bcl-2 family proteins have also been shown to regulate the cross talk between autophagy and cell death (Fig. 14.5).

Beclin-1 is a BH3-only protein that interacts with Bcl-2, Bcl-xL, Bcl-w, Bcl-B, and Mcl-1, but does not interact with the prodeath BH3-only members of the Bcl-2 family (Pattingre et al. 2005; Erlich et al. 2007; Maiuri et al. 2007a; Robert et al. 2012).

Much like other BH3-only proteins, Beclin-1 interacts with Bcl-2, Bcl-xL, and Mcl-1 via its BH3 domain, and mutations of this domain or of the BH3-receptor domain in antiapoptotic Bcl-2 family members prevent this interaction (Feng et al. 2007; Maiuri et al. 2007a; Oberstein et al. 2007). This interaction inhibits the Beclin-1-mediated induction of autophagy under nutrient-sufficient conditions in a location-dependent manner. Although Bcl-2, Bcl-xL, and Mcl-1 are mainly localized to the mitochondria, where they serve to inhibit MOMP (see Sect. 14.4.2), only ER-localized Bcl-2 family members can inhibit starvation-induced autophagy (Pattingre et al. 2005; Maiuri et al. 2007a). Although the reasons for the subcellular localization-dependent ability of Bcl-2 family members to

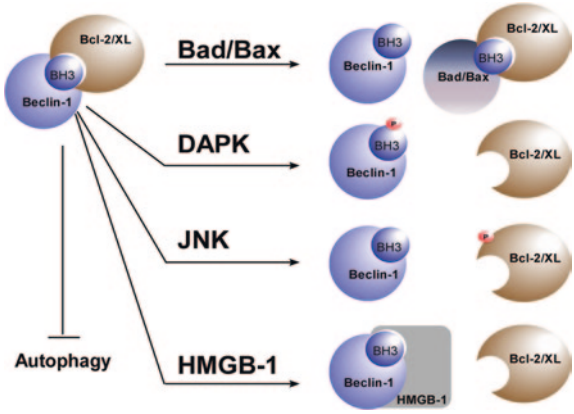

Fig. 14.5 Beclin-1–Bcl-2 family interactions. In a rich medium, Beclin-1 is bound by Bcl-2 or Bcl-xL, and its ability to initiate autophagy is inhibited. Under stressful conditions, several mechanisms mediate the disruption of this interaction, thus allowing autophagy to occur. These mechanisms include competition with Bad and Bax for Bcl-2/Bcl-xL binding, death-associated protein kinase (*DAPK*)-mediated phosphorylation of the BH3 domain of Beclin-1, c-Jun N-terminal kinase (*JNK*)-mediated phosphorylation of the nonstructured loop of Bcl-2, and high mobility group box 1 (*HMGB-1*) displacement of Bcl-2 by a direct interaction with Beclin-1

inhibit the autophagic function of Beclin-1 remain unclear, recent findings suggest the involvement of the novel Bcl-2 binding partner nutrient-deprivation autophagy factor 1 (NAF-1), which appears to stabilize the Bcl-2-Beclin-1 interaction at the ER (Chang et al. 2010). Importantly, NAF-1 knockdown results in disruption of the Bcl-2–Beclin-1 interaction and induction of autophagy, further demonstrating the inhibitory role of Bcl-2 family members in Beclin-1-mediated autophagy (Chang et al. 2010). During starvation or other stress conditions, however, Bcl-2 and Bcl-xL must be displaced from Beclin-1 to permit autophagy to occur (Pattingre et al. 2005; Wei et al. 2008).

The dissociation of this complex can be achieved through c-Jun N-terminal kinase (JNK)-mediated phosphorylation of Bcl-2, death-associated protein kinase (DAPK)-mediated phosphorylation of Beclin-1, translocation of the nuclear protein HMGB1 to the cytosol, or competition with other BH3-only proteins for Bcl-2 binding (Maiuri et al. 2007b; Wei et al. 2008; Zalckvar et al. 2009b; Luo and Rubinsztein 2010); (Kang et al. 2010; Tang et al. 2010a). Nutrient deprivation results in the activation of the stress-induced mitogen-activated protein kinase JNK, which phosphorylates three residues in the regulatory loop of Bcl-2, thus disrupting its interaction with Beclin-1 (Wei et al. 2008). This process is essential for the induction of autophagy under nutrient-poor conditions, as cells with either mutations of these phosphorylation sites or JNK deficiency do not undergo starvation-induced autophagy. Moreover, the expression of constitutively active JNK is sufficient to induce autophagy in complete medium in cells expressing wild-type Bcl-2, but not in cells expressing a nonphosphorylatable Bcl-2 mutant (Wei et al.

2008). JNK has also been implicated in the induction of autophagy in response to ER stress, oxidative stress, cancer drugs, and stimulation through the death receptor Fas; however, it has yet to be shown whether JNK mediates phosphorylation of Bcl-2 under these circumstances (Ogata et al. 2006; Zhang et al. 2008; Li et al. 2009b; Wu et al. 2009). In addition to this stress-induced phosphorylation of Bcl-2 by JNK, dissociation of the Bcl-2/Bcl-xL–Beclin-1 complex and the subsequent induction of autophagy can be achieved by DAPK-mediated phosphorylation of Beclin-1 on its BH3 domain (Zalckvar et al. 2009a, b). Cells expressing a mutant Beclin-1 that mimicked the phosphorylation of Thr119 within the BH3 domain displayed decreased Bcl-xL–Beclin-1 binding and enhanced autophagosome formation, whereas a nonphosphorylatable Beclin-1 mutant displayed enhanced Bcl-xL binding. DAPK, which functions as a tumor suppressor, has been implicated in both death-receptor-mediated apoptosis and ACD (Bialik and Kimchi 2006); however, it has yet to be shown whether DAPK-mediated dissociation of the Bcl-2/Bcl-xL–Beclin-1 complex serves a prodeath or prosurvival function. Thus, the enhanced autophagy observed in DAPK-overexpressing cells may protect cells against DAPK-mediated apoptosis. An additional stress-related signal that triggers the dissociation of the Bcl-2/Bcl-xL–Beclin-1 complex involves the translocation of HMGB1 to the cytosol. Recent studies have demonstrated that HMGB1 translocation also occurs in response to autophagic stimuli. Moreover, translocated HMGB1 displaces Bcl-2 by interacting directly with Beclin-1, and decreased starvation-induced autophagy has been observed after inhibition of the cytosolic translocation of HMGB1 (Tang et al. 2010c; Kang et al. 2011). Furthermore, the Bcl-2–Beclin-1 complex can be disrupted by proapoptotic, BH3-only proteins. Both Bad and Bax can disrupt the interaction between Bcl-2 and Beclin-1; however, these proteins have opposite effects on Beclin-1-mediated autophagy (Maiuri et al. 2007a; Luo and Rubinsztein 2010). Bad reverses the Bcl-2-mediated inhibition of autophagy, whereas overexpression of Bax reduces autophagy (Maiuri et al. 2007a); (Luo and Rubinsztein 2010). Although these results seem to suggest that Bad and Bax may have opposing functions, the ability of Bax to inhibit autophagy may be related to its ability to stimulate the degradation of Beclin-1 rather than to its ability to disrupt the Bcl-2–Beclin-1 complex (Luo and Rubinsztein 2010). Intriguingly, whereas the Bcl-2/Bcl-xL–Beclin-1 interaction inhibits Beclin-1-dependent autophagy; it does not affect the antiapoptotic function of Bcl-2 (Pattingre et al. 2005; Boya and Kroemer 2009; Ciechomska et al. 2009a, b). Several possible explanations have been proposed to explain why Bcl-2/Bcl-xL–Beclin-1 binding has no effect on apoptosis, most of which involve the fact that the affinity of Bcl-2 for Beclin-1 is relatively weak in comparison with its affinity for other BH3-only proteins; however, further experiments are necessary to fully elucidate the function of the Bcl-2/Bcl-xL–Beclin-1 interaction in apoptosis (Boya and Kroemer 2009; Ciechomska et al. 2009a, b). It has recently been demonstrated that Atg12 is a positive mediator of mitochondrial apoptosis (Rubinstein et al. 2011) by binding to the prosurvival proteins Bcl-2 and Mcl-1. This binding requires a BH3-like motif in Atg12, and occurs independently of Atg5.

Although these findings overwhelmingly suggest that Beclin-1 is involved in linking autophagy and apoptosis, recent findings suggest that autophagy can also

occur in the absence of some of the key autophagy proteins, including Beclin-1 (Codogno et al. 2011). Thus, it remains unclear whether Beclin-1 serves as a bridge between apoptosis and autophagy under all circumstances.

14.5 Autophagy in Cancer

The role of autophagy is complex, and several recent reviews have discussed different aspects from basic research to therapeutic targeting (Cheong et al. 2012; White 2012; Liu and Ryan 2012; Rubinsztein et al. 2012). The initial link between cancer and autophagy was made on the basis of the observation that *BECN1* (the gene encoding Beclin-1) is monoallelically deleted in around 50 % of sporadic breast, ovarian, and prostate cancers (Aita et al. 1999; Liang et al. 1999). Forced expression of Beclin-1 in a breast cancer cell line, which increased the rate of autophagy, decreased the tumorigenicity of the cells when it was injected into mice (Liang et al. 1999). The prevailing current view is that autophagy functions both as a tumor-suppressor pathway that prevents tumor initiation and as a prosurvival pathway that helps tumor cells cope with metabolic stress and resist death triggered by chemotherapeutic agents (Janku et al. 2011).

14.5.1 Tumor Initiation

There is general consensus that autophagy suppresses tumor initiation (Fig. 14.6). Genetic deletion of *BECN1* is associated with susceptibility to breast, ovarian, and prostate cancer in humans (Aita et al. 1999; Liang et al. 1999), and an increase in spontaneous malignancies (lung cancers, hepatocellular carcinomas, and lymphomas) in mice, like its protein partners, including Ambra1, Bif-1, and UVRAG (Qu et al. 2003; Yue et al. 2003). Mice deficient in Atg4C, a cysteine protease required for the processing of LC3, show increased susceptibility to chemically induced fibrosarcomas (Marino et al. 2007), and mice in which *Atg5* or *Atg7* has been deleted develop benign liver tumors (Takamura et al. 2011). Besides this direct evidence that autophagy, gene mutation, or deficiency of autophagy promotes tumorigenesis, oncogenic-signaling activation and the suppression of autophagy have been found to overlap (reviewed in Morselli et al. 2011a). Several tumor suppressor genes, including *PTEN*, *TSC1*, *TSC2*, and *LKB1*, stimulate autophagy either through their inhibitory effects on mTOR or their activation of the ULK1 autophagy complex, or both. Conversely, mTOR-activating oncogenic signals, such as oncogenic receptor tyrosine kinases, class I PI3K, and Akt, inhibit autophagy. Other tumor suppressors, such as DAPK and $p19^{ARF}$ and oncogenes such as Bcl-2 either upregulate or downregulate autophagy through their effects on Beclin-1 (Pimkina et al. 2009; He and Levine 2010).

However, in human cancers, p53, the most commonly mutated tumor suppressor gene, has both positive effects on autophagy as a result of its nuclear transcriptional

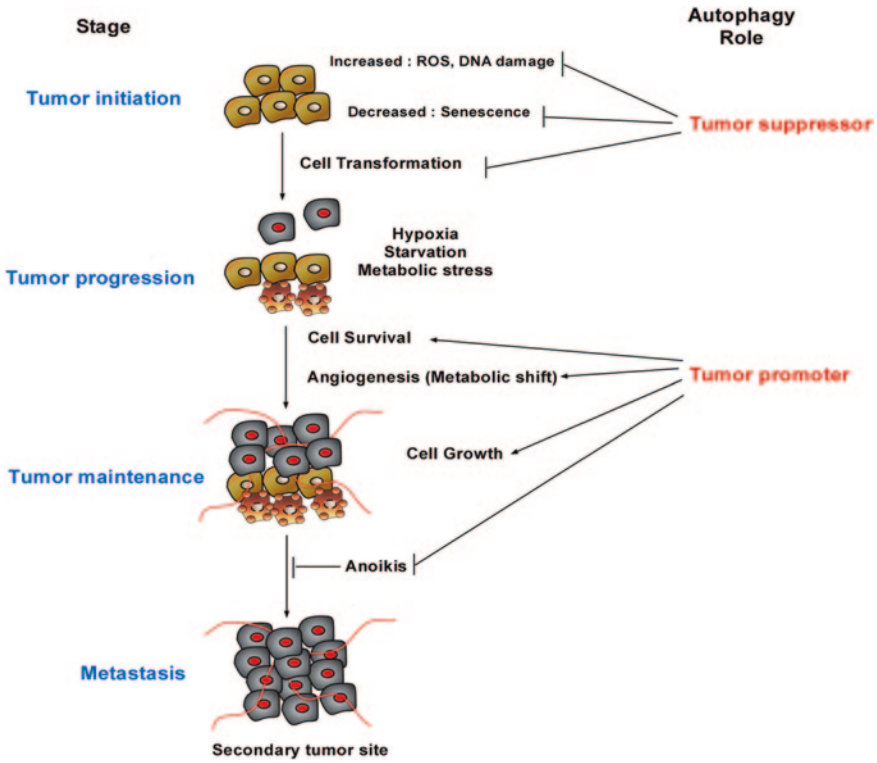

Fig. 14.6 The complex and context-dependent role of autophagy in cancer. During tumor initiation, autophagy acts as a tumor suppressor because it constitutes a barrier to cell transformation by reducing cell proliferation, ROS production, and DNA damage. Autophagy also plays a role in preventing cell transformation by inducing senescence. In contrast, in established tumors, autophagy may act as a tumor promoter. Indeed, high levels of autophagy have been shown to increase cancer cell survival under conditions of starvation, hypoxia, and metabolic stress—conditions often observed in primary tumors. The cancer cells subsequently become dependent on autophagy to sustain growth, either because it provides the basic building blocks required for anabolic reactions or because ultimately autophagy sustains tumor angiogenesis, or both. Autophagy may also promote tumor cell metastasis by protecting epithelial cancer cell from anoikis

activity, and negative effects through its cytoplasmic actions, including an interaction with the human orthologue of yeast Atg17, FIP200 (Tasdemir et al. 2008; Morselli et al. 2011b). It has been shown that mutant forms of p53 that accumulate in the cytoplasm in human cancers suppress autophagy (Morselli et al. 2008). In the same way, Ras oncogenes (H-*ras* or K-*ras*) have dual functions in autophagy regulation, both inhibiting autophagy by activating class I PI3K (Furuta et al. 2004) and stimulating autophagy, perhaps through effects on Raf-1/ERK signaling, RalB signaling, or upregulation of BNIP3, Noxa, or Beclin-1 expression (Wu et al. 2011; Elgendy et al. 2011; Kim et al. 2011b). Three recent studies have shown that autophagy is essential for Ras-induced malignant cell transformation (Guo et al. 2011; Kim et al. 2011c; Lock et al. 2011). In contrast, another study has shown that autophagy

gene knockdown increased clonigenic survival in cells expressing oncogenic Ras (Elgendy et al. 2011). It is not yet known whether the requirement for autophagy in Ras-driven transformation observed in most studies is a unique feature of cells with Ras activation, or is also observed in the context of other oncogenes. It is possible that Ras alters metabolism in a unique manner that generates a specific requirement for autophagy in cell proliferation and/or cell survival. One hypothesis is that mitochondrial functional loss mediated by oncogenic-Ras-induced mitophagy overcomes the cellular energy deficit resulting from glucose insufficiency (Kim et al. 2011b).

Although the precise mechanisms by which autophagy mediates tumor suppression are not completely understood, several pathways probably play a contributory role (reviewed in Morselli et al. 2011a). Many of these relate to the ability of autophagy to remove damaged organelles, especially mitochondria, which generate ROS, which in turn promote genotoxic stress and proinflammatory/protumorigenic signaling. Tumors formed by autophagy-deficient cells (with biallelic loss of *Atg5* or monoallelic loss of *Becn1*) display genomic instability and DNA damage, which is in part mediated by ROS (Mathew et al. 2007b; Mathew et al. 2009).

Another important mechanism by which basal autophagy may prevent cancer is by controlling the levels of selective autophagy adaptor proteins, such as p62, that function in protumorigenic signaling. The size of liver tumors in $Atg7^{-/-}$ mice is reduced by simultaneous deletion of p62 (Takamura et al. 2011); gene targeting of p62 reduces anchorage-independent growth of human hepatocellular carcinoma cells (Inami et al. 2011); and $p62^{-/-}$ mice are resistant to Ras-induced lung carcinomas (Duran et al. 2008). The accumulation of p62 in autophagy-deficient cells promotes tumorigenesis through a mechanism postulated to involve its role as a scaffold protein that is involved in the activation of the transcription factors NF-κB and NRF2 (Duran et al. 2008; Inami et al. 2011). In addition, p62 interacts with and activates the mitogenic signaling and autophagy-suppressive molecule mTOR (Duran et al. 2011). A p62-interacting protein, SMURF1, which is also involved in mitophagy (Orvedahl et al. 2011), is amplified in pancreatic carcinomas (Loukopoulos et al. 2007; Birnbaum et al. 2011; Kwei et al. 2011). Finally, autophagy is also required for oncogene-induced senescence, an irreversible arresting of the cell cycle that limits the proliferation of transformed cells (Young et al. 2009). It has been demonstrated that autophagy is activated during senescence induced by oncogenes and DNA damage, and depletion of Atg genes helps the transformed cells to escape the senescence phenotype. Thus, autophagy may play a role in restricting cell proliferation of transformed cells via senescence.

14.5.2 Tumor Progression

There is increasing evidence that autophagy may be necessary for the progression and survival of tumors (Fig. 14.6). The retention of a wild-type allele of *Becn1* in tumors arising in mice with heterozygous deletion of *Becn1* (Qu et al. 2003) provided one of the first genetic clues that tumor cells may need to retain autophagy for malignant progression to occur (Edinger and Thompson 2003). This concept has been further

confirmed by the recent observation that hepatic deletion of *Atg7* or *Atg5* results in benign, but not malignant hepatic tumors (Takamura et al. 2011). However, an alternative explanation is that the background mouse strain may not develop the additional mutations required for malignant transformation. For example, *Becn1* heterozygous-deficient mice develop preneoplastic mammary lesions, but not mammary carcinomas, even though *Becn1* allele loss is associated with breast cancer in humans and with other malignancies in mice (Liang et al. 1999; Qu et al. 2003; Yue et al. 2003). Nonetheless, several other studies also suggest that autophagy has a role in promoting the growth of established tumors in vivo. For example, monoallelic loss of *Becn1* almost completely blocked macroscopic renal tumor formation in $Tsc2^{+/-}$ mice (Parkhitko et al. 2011), and delayed tumor development in $ATM^{-/-}$ mice (Valentin-Vega et al. 2012). Biallelic deletion of *Atg5* or *Atg7* impaired tumor growth of Ras-transformed immortalized baby mouse kidney epithelial cells in nude mice (Guo et al. 2011) and *ATG5* short hairpin RNA impaired tumor growth of human pancreatic ductal adenocarcinoma cells in a mouse xenograft model (Yang et al. 2011).

The concept underlying these phenotypes is that autophagy gives tumor cells a survival advantage by enabling them to overcome the metabolic stress inherently present in the tumoral microenvironment. Consistent with this notion, autophagy is induced by cellular stress, including nutrient, growth factor, and oxygen deprivation, and helps to maintain the survival of normal cells, and organisms, but also of tumor cells, in these settings (reviewed in Rabinowitz and White 2010).

The pro-oncogenic function of autophagy in established cancer may be context-dependent; not all data are consistent with a pro-oncogenic role for autophagy in established tumors. While autophagy upregulation, in part mediated by hypoxia-induced effects of transcription factor 1α on BNIP3 (Bellot et al. 2009), may promote tumor cell survival in hypoxic regions within tumors (reviewed in Mathew et al. 2007a)), a recent study has shown that autophagy may also protect against hypoxia-stimulated tumor growth by reducing tumor angiogenesis (Lee et al. 2011). In a mouse melanoma xenograft model, *beclin 1* heterozygous-deficient mice display a more aggressive tumor phenotype with increased angiogenesis under hypoxic conditions through a mechanism postulated to involve the upregulation of HIF-2α (but not of HIF-1α). Furthermore, several studies involving clinical and pathology observations have shown a correlation between levels of Beclin 1 expression and cancer prognosis; low levels are associated with a worse cancer prognosis in gastric cancer (Maycotte et al. 2012), colorectal cancer (Li et al. 2009a), pancreatic cancer (Kim et al. 2011a), esophageal cancer (Chen et al. 2009), chondrosarcoma (Kim et al. 2011a), and breast cancer (Maycotte et al. 2012), whereas high levels of Beclin 1 expression are associated with improved survival in high-grade gliomas (Pirtoli et al. 2009), hepatocellular carcinomas (Ding et al. 2008), and B cell lymphomas (Huang et al. 2011). Although it is not known whether low levels of Beclin-1 expression are directly correlated with low levels of autophagy in these tumors, these findings highlight the need for further careful analyses of the relationship between autophagy levels and tumor progression in different types of tumors.

Autophagy may also promote tumor cell metastasis by protecting epithelial cells against anoikis, a form of programmed cell death induced by the detachment

of anchorage-dependent cells from the extracellular matrix (EM). Indeed, in order to metastasize, tumor cells have to detach themselves from the EM and therefore to survive anoikis. It has been observed that autophagy is induced during the separation of epithelial cells from EM and protects these cells against anoikis. RNA interference-mediated depletion of Atg proteins enhances apoptosis and reduces clonogenic recovery after anoikis (Fung et al. 2008).

14.5.3 Autophagy in "Cancer Stem Cells"

Accumulating evidence supports the existence of a "cancer stem cell" (CSC)/"tumor-initiating cell" system in which malignant cancers comprise heterogeneous populations that are organized in a hierarchical differentiation model governed only by a small subset of cells exhibiting proprieties similar to those of stem cells and are responsible for carcinogenesis, tumor maintenance and/or recurrence, distant metastasis, and resistance of tumors to standard cancer therapies, including chemotherapy and radiotherapy. Today, after pioneering work in leukemia, CSCs/tumor-initiating cells have been identified and isolated from several types of cancers, including brain tumors, breast cancer, prostate cancer, colon cancer, and melanoma (Al-Hajj et al. 2003; Singh et al. 2003). These initial studies show that cell-surface marker profiles, such as $CD44^{high}/CD24^{-/low}$ epithelial-specific antigen positive, can be used to isolate subpopulations in which CSCs/progenitor cells have been enriched in breast cancer. Aldehyde dehydrogenase 1 activity offers an alternative candidate marker of stem/progenitor cell population in both the normal mammary gland and mammary carcinomas (Ginestier et al. 2007; Charafe-Jauffret et al. 2010). Other key features of CSCs include the formation of tumor spheres in low-adherence cultures in serum-free medium, high tumorigenicity, and multidrug resistance (Pardal et al. 2003; Clement et al. 2007; Gangemi et al. 2009). Eradication of the CSC population is a major clinical challenge in cancer therapy. Emerging data indicate that autophagy pathways play an important role in the survival, self-renewal, and differentiation of both stem cells and CSCs (Cufi et al. 2011; Mortensen et al. 2011; Oliver et al. 2012; Salemi et al. 2012). We have recently shown that autophagy is essential for the maintenance and tumorigenicity of CSCs in breast cancer (Gong et al. 2012). The expression of Beclin-1 is higher in mammospheres established from human breast cancers or breast cancer cell lines (MCF-7 and BT474) than in the parental adherent cells. As a result, autophagic flux is more robust in mammospheres. Furthermore, the basal and starvation-induced autophagy fluxes are also higher in the aldehyde dehydrogenase 1 positive population derived from mammospheres than in the bulk population. It has been demonstrated that Beclin-1 is critical for CSC maintenance and tumor development in nude mice, whereas its expression limits the development of tumors that are not enriched with breast CSCs/progenitor cells. We found that decreased survival in autophagy-deficient cells (MCF-7 *ATG7* knockdown cells) during detachment does not contribute to an ultimate deficiency in mammosphere formation. This study demonstrated that a prosurvival autophagic

pathway is critical for CSC maintenance, and that Beclin-1 plays a dual role in tumor development. This research indicates a possible new strategy for killing CSCs, involving inhibition of the autophagy flux, thus encouraging the development of therapeutic molecules targeting the autophagy flux of the CSC/progenitor phenotype, which is more susceptible to its inhibition.

14.6 Conclusion and Future Perspectives

As our understanding of the biological functions of autophagy increases, the involvement of autophagy in the cell death and cell survival processes becomes a critical point of concern. Here we have attempted to review some of the emerging issues surrounding the relationship between autophagy, apoptosis, and necrosis and the role of autophagy in cancer; autophagy first serves as a cell-survival mechanism, via its role in suppressing apoptosis and necrotic cell death. We have reviewed the molecular cross talk between autophagy and cell death. Studies deciphering this molecular cross talk could help to elucidate the relationship between autophagy and a cell-death-dedicated mechanism (Bialik et al. 2010). A recent proteomic approach has revealed that hundreds of interactions occur between human proteins and the core autophagic machinery (Behrends et al. 2010), suggesting that some hitherto unknown aspects of autophagy regulation could lead to better understanding of its place in cell functioning, including cell death. Although the translation of basic knowledge of the autophagic process into a therapeutic intervention is a lengthy process, it is reasonable to speculate that autophagy modulation should be viewed as a potential therapeutic approach in cancer. There are several phase I/II clinical trials in progress using lysosome-inhibitor drugs, chloroquine, rapamycin, or hydroxychloroquine, alone or in combination with chemotherapy to treat a range of hematological and solid tumors (Rubinsztein et al. 2012). However, it is not possible to exclude the possibility that the beneficial effect of these drugs could be independent of their blocking effect on the autophagic pathway. The development of more specific autophagy modulators, both for therapeutic investigations and to allow acute modulation of this process for cell biology and physiological studies, is a major challenge for the future.

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Chapter 15
The Duality of Stem Cells: Double-Edged Sword in Tumor Evolution and Treatment

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Abstract Cancer is a disease of the gene, caused by genetic and epigenetic mutations, resulting in the development of malignant cells. Previous concepts of the monoclonal origin of tumors and the stochastic nature of carcinogenesis are being challenged by hierarchical/plasticity models that predict the existence of tumor-initiating and tumor-propagating cells, which have stem cell properties and are thus highly resistant to therapy. It may therefore be postulated that cancer is a stem cell disease. One of the actively investigated tumors of cancer stem cell origin is glioma, one of the commonest primary brain tumors in adults. In this chapter we shall focus on glioblastoma multiforme (GBM), glioma's most malignant stage, characterized by enhanced, single-cell infiltration into normal brain. The invasive cell phenotype is the major obstacle for successful therapy, as highly invasive cells that can migrate from the bulk tumor mass cannot be removed by any currently available therapy. Invasion is a complex, stepwise process, initiated at various levels—from gene activation to the level of protein post-translational modification. At the phenotype level, the process is reminiscent of the epithelial to mesenchymal transition in carcinoma malignization, which includes the activation of the so-called migratome, possibly overlapping with proteases, i.e. a cancer

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degradome. On the basis of the hierarchical/plasticity model of carcinogenesis, the cells undergoing epithelial to mesenchymal transition may also have stem/progenitor cell characteristics. Other types of stem cells associated with tumors are infiltrating haematopoietic stem cells, endothelial progenitor cells and mesenchymal stem cells (MSCs), comprising the tumor microenvironment. Their role and differentiation pathways in tumor niches are the subject of intensive investigations, and unravelling the mechanisms underlying MSC-mediated modulation of tumor behaviour via paracrine and direct interactions with GBM stem cells in vitro may solve the dispute regarding their antimalignant activity, which is currently contradictory to the hypothesis of a possible mesenchymal origin of GBM stem cells. By the same token, MSCs may be novel therapeutic vectors for drug delivery, which are badly needed. Cell therapies using stem cells are promising owing to their selective tropism towards tumors. In particular, autologous MSCs are considered promising at least in some tumors, especially owing to their immunomodulatory potential. However, MSC therapy may have adverse effects, since the role of MSCs in tumor progression has been evidenced. Combined cell-based drug and gene delivery with anti-invasive strategies could therefore contribute to enhanced efficacy of conventional therapeutic approaches in glioblastoma treatment. In this chapter we shall thoroughly revisit the advantages of and obstacles in recent preclinical trials using stem cells for treating brain tumors.

Keywords Brain tumors • Cancer stem cells • Cell therapy • Epithelial to mesenchymal transition • Glioblastoma multiforme • Mesenchymal stem cells • Proteases • Tumor microenvironment

15.1 Introduction

In spite of extensive investigations and expanded translation of research into clinical trials, cancer remains a disease where even after treatment there is a lasting threat of relapse. Still, the rapid advances in cell biology and biological techniques, particularly after including broader aspects of whole genome, transcriptome, proteome and other 'omics' approaches, are now providing us with answers which were beyond our reach 10 years ago. That is the reason why despite increased cancer incidence, the mortality from the disease is decreasing and increased survival of patients after diagnosis is observed.

Carcinogenesis is a multistep process where cancer cell initiation is followed by propagation, which results in proliferation of damaged cells and further processes, which are generally called tumor progression. In the first part of this chapter, we shall focus mainly on the cancer-initiating cells that are capable of sustaining tumor growth, called cancer stem cells (CSCs), but which are not necessarily the first damaged (genetically deregulated) cells. Their differentiation into highly proliferating and malignant cancer cells depends on their additional genetic changes and the adaptability of transformed cells to the forming tumor microenvironment. The

process, reminiscent of Darwinian evolution principles, is called cancer evolution and may proceed via various pathways. Tumor progression is believed to be driven by the development of a sustainable variability between different tumor cell subpopulations, conditioned by the tumor microenvironment, moving from initial host rejection to a host-supporting environment, assisting tumor growth and invasion. The interactions between tumor cell subpopulations and host cells seem to be crucial during this process and have been the subject of intensive investigation (Mueller and Fusenig 2004). The cellular and structural environment does not only condition the tumor, but is also altered and conditioned by the growing mass of heterogeneous tumor cells in the process of so-called cellular cross talk. During the ensuing decade, this notion was accepted and extended, revealing that the biology of tumors can no longer be understood simply by studying only numerous traits of the cancer cells but instead must include the contributions of the tumor microenvironment comprising the so-called stromal cells to tumorigenesis as well as to metastasis formation (Hanahan and Weinberg 2011).

With respect to the origin and evolution of tumors, the theory of the hierarchical model of tumorigenesis has been widely accepted. This states that only a small fraction of tumor cells—the CSCs, are capable of initiating tumor growth or renewing the tumor in the same or another organ after incomplete surgical removal (Reya et al. 2001; Wicha et al. 2006; Visvader and Lindeman 2008). When injected orthotopically, CSCs were phenotypically characterized as capable of self-renewal, asymmetric division and tumor formation in animal models. They most likely originate from cells of various types within the diseased tissue (Al-Hajj and Clarke 2004; Li and Neaves 2006; Hadjipanayis and Van Meir 2009; Van Meir et al. 2010) via postulated cellular events such as cell fusion and horizontal gene transfer (Bjerkvig et al. 2005). CSCs are also highly resistant to chemotherapy or radiotherapy (Signore et al. 2013), making current cancer treatments failure-prone. The CSC theory, cancer initiation, progression, invasion, and possible treatments will be explained in this chapter and exemplified by the most malignant and abundant brain tumor, glioma.

The key issue of cancer treatment remains CSCs, which represent the invisible moving targets that can acquire the migratory phenotype, enabling them to dislocate and regrow distant tumors (metastasis). Presumably, their immediate progenitors may also have the same invasive potential (Dalerba and Clarke 2007; Brabletz 2012). The transition from stationary to migratory type is also associated with the process of epithelial to mesenchymal transition (EMT), which may occur at least in epithelial CSCs as discussed later. The question remains whether this actually applies to glioma stem cells and to what extent EMT here involves reversible epigenetic and/or genetic changes in contrast to the one-way tumorigenesis.

Recent therapeutic approaches to cancer seem to follow multiple targets, which are not only CSCs, but also stromal cells. The cellular stroma comprises the cells of the immune system, extracellular matrix (ECM) supporting fibroblasts and endothelial cells. All these may also originate from progenitors of infiltrated normal stem cells—haematopoietic stem cells and mesenchymal stem cells (MSCs), mostly originating from bone marrow, during the in situ differentiation process.

The interactions of MSCs with the evolving tumor cells and transformed tissue are discussed in the second part of this chapter. The novel treatment strategies appearing on the horizon and using MSCs as Trojan horses by modifying them, for example, with oncolytic viruses carrying the already known tumor cell killing agents or using other gene silencing/enhancing molecular strategies to eradicate tumor (stem) cells are discussed in the last part of this chapter.

15.1.1 Cancer Origin

Cancer comprises various diseases (more than 400 have been described in the literature), and although carcinogenesis follows similar principles in different tissues, both the molecular pathway and the cellular origin are rather different in many of them. It is often claimed that cancer is primarily an environmental disease because of the 90–95 % impact of the environment and only 5–10 % impact of heredity (Anand et al. 2008). The evidence indicates that of all cancer-related deaths, almost 25–30 % are due to tobacco, as many as 30–35 % are linked to diet, about 15–20 % are due to infections, whereas the remaining deaths are due to other factors such as radiation, stress, physical activity and environmental pollutants. On the other hand, endogenous hormonal and metabolic imbalances in the human organism independently of clearly defined external factors cause disturbances, adding to or being part of the genetic predisposal to carcinogenesis of an individual (Tysnes and Bjerkvig 2007). The latter has been revealed over the past few years on the basis of meta-analyses of cancer transcriptomics and growing knowledge of regulation of genome expression by non-coding RNA, both paving the way to what we call personalized oncology, which will hopefully be used in the future (Chin and Gray 2008).

The hallmarks of cancer were elaborated by Hanahan and Weinberg (2000) at the beginning of the millennium and they comprise autonomous growth, i.e. self-sufficiency of growth signals, insensitivity to physiological normal cell growth inhibition, alteration of the cell cycle resulting in limitless replicative potential evading apoptosis, sustained angiogenesis induction and, most importantly, tissue invasion and metastasis. The latter is of utmost importance considering that about 90 % of patients have tumor growth that is discovered at sites remote from the location of the primary tumor. Cancer cells are considered malignant as they can be disseminated either locally or systemically via lymph or blood flow, versus benign cells, which are self-limited, and do not invade or metastasize. After a decade Hanahan and Weinberg (2011) updated the cancer concepts and concluded that we have witnessed remarkable progress towards understanding each of the previously described hallmarks. Apparently, new key properties were described, such as genome instability, which generates the genetic diversity, and inflammation, which fosters multiple functional changes. In addition, two novel emerging hallmarks were added to the list, i.e. the altered energy metabolism and the evasion of the host immune response. The most important recent concept that is gaining worldwide recognition addresses besides cancer cells within the tumor also

recruited normal cells that may contribute to the acquisition of hallmark traits by creating the tumor permissive microenvironment. Further recognition of the tumor cell/normal cell cross talk via their paracrine or direct exchange of signalling molecules will have a great influence on new means to treat human cancer.

A stepwise process of cancer malignancy evolution underlies the above-mentioned hallmarks, as it selects for genetically unbalanced cells, with altered metabolic demands and producing metabolites, which affect the tumor microenvironment—the stroma. This, on one hand, sustains the presence of cancer cells, while on the other hand, causes alterations that facilitate detachment of cancer cells. Macroscopically, a visible tumor first appears as a mass of proliferating cells, from which later defined circulating tumor cells (CTCs) (Sahai 2007) may spread into the blood and as disseminating tumor cells (DTCs) become resident in bone marrow (Bednarz-Knoll et al. 2011). These when spread as micrometastasis may develop into solid macrometastasis at the selected sites and fatally disturb the function of various organs.

15.1.2 Molecular Initiation and Progression

A monoclonal origin of tumors has long been an accepted principle of cancer evolution, postulating that cancer initiation is a molecular event resulting from the ultimate cellular response to various carcinogens. Later concepts of the so-called stochastic model of cancer evolution then postulated that both processes, the response to carcinogens and increased accumulation of mutations, which increase the genetic instability of tumor cells and arise because of unsuccessful protection against the external or internal carcinogens, actually run in parallel (Hanahan and Weinberg 2000). Moreover, the model predicts that the joint processes lead to 'Darwinian evolution', based on the survival of the fittest—in this case the most adapted transformed cell to the newly emerging intratumor environment. By this cellular evolution, the variability of tumor phenotype in different tissues, as well as the appearance of the metastatic phenotype—the cell's ability to disseminate and form the original tumor at a distant site in an organism, may be explained. A more recent review by Tysnes and Bjerkvig (2007) proposed two alternative events that may lead to cancer development: First, cancer may be initiated by all known external carcinogens (chemicals, radiation, microorganisms and viruses), causing mutations and clonal selection that would increase genetic and chromosomal instability, resulting in transformation of cells into malignant aneuploid derivates lacking growth control. Alternatively, cancer may be initiated by the prime formation of aneuploidy, which may still be induced by the external carcinogens mentioned, yet more likely this initial aneuploidy results from disturbed hormonal homeostasis and inflammation mediated by intrinsic factors. This second alternative may more likely result in events such as cell fusion, transdifferentiation, horizontal gene transfer and epigenetic changes. These may then trigger genetic events, meaning that the mutations may occur rather late in the tumor development process. During tumor progression, the sequence of events may also be interrelated.

Tumor formation is thus rather complex and not always a linear process, which is regulated by several classes of genes, controlled by oncogenes and tumor suppressor genes, being irreversibly altered as a result of initiation and promotion events and increased proliferation of transformed/damaged cells, further triggering the so-called metastatic genes. By their functional activity, these are classified as gatekeeper, caretaker and landscaper genes (Hanahan and Weinberg 2011). Hereditary or acquired abnormalities in these regulatory genes can predispose to the above-mentioned events. As such, the events leading to uncontrolled cell growth and the development of cancer can be triggered earlier in the lifetime compared with usually increased cancer incidence after the age of 50–55 years (Chin and Gray 2008; Cancer Research UK 2012).

15.1.3 Invasion and the Epithelial to Mesenchymal Transition–Mesenchymal to Epithelial Transition Hypothesis: Cancer Cells as Moving Targets

Tumor progression is associated with repeating alternations of tumor cell phenotypes between a fast proliferating phenotype, enhancing the growth of neoplastic tissue, and the migratory phenotype responsible for tumor dissemination and metastasis. The migratory phenotype has recently been observed to simultaneously acquire traits of stemness and dedifferentiation (Borovski et al. 2009; Charafe-Jauffret et al. 2009; Thiery et al. 2009). The molecular events (Biddle et al. 2011) underlying these alternative processes have recently been summarized in a review by Brabletz (2012) and are referred to as the well-known process of EMT and the reverse process of mesenchymal to epithelial transition (MET). Besides, in cancer (stem) cells, both processes are known to occur and alternate also during tissue and organ evolution.

It is now well accepted that aberrant activation of the EMT-stemness programme, which is triggered by environmental factors such as inflammation and hypoxia, drives the abnormal motility of cancer cells. It causes the activation of the so-called metastasis virulence factors (Nguyen et al. 2009)—the genes which enhance dissemination at all levels of the metastatic cascade. Moreover, the same principle may be applied to normal bone-marrow-derived MSCs (BM-MSCs) and may enable migrating CSCs to enter the blood vessels and disseminate as CTCs that seed into distant organs and form micrometastases and macrometastases (Brabletz et al. 2005; Thiery et al. 2009). Moreover, as described in Sect. 15.1.1, a subset of CTCs may even colonize the bone marrow and become DTCs, which have a confirmed bad prognostic impact on patient survival (Bednarz-Knoll et al. 2011). When CTCs colonize other permissive organs and form micrometastases, they may initially become quiescent or even senescent (Aguirre-Ghiso 2007). After a variable time frame and depending on the microenvironmental stimuli at these secondary sites, these quiescent CTCs/DTCs may regain the ability to initiate growth and proceed from micrometastases to macrometastases, which therefore represents the ultimate rate-limiting process in malignization.

As already indicated, the paracrine cross talk between the target organ, infiltrating cells and the primary tumor cells is crucial for tumor development. One current theory proposes that systemic factors, secreted by the primary tumor, attract MSCs from the bone marrow to distant organs to form a so-called premetastatic niche, which then allows the seeding of CTCs (Visvader and Lindeman 2008; Borovski et al. 2011; Brabletz 2012). Numerous signals may shape such a premetastatic niche, stimulating differentiation of MSCs to fibroblasts and endothelial cells, this way making possible their growth, vascularization and finally the colonization of niche by CTCs (Psaila and Lyden 2009). Here, the process of MET allows a kind of redifferentiation of DTCs to a stationary phenotype of CSCs which may play a crucial role in macrometastases development in many differentiated carcinoma types. Understanding all these processes would explain the long-known 'seed and soil hypothesis,' posted by Sir Percival Pott in 1885 and referring to metastatic organ tropism. The crucial molecular players associated with these processes are various cytokines—interleukins and chemokines as well as growth factors, in particular vascular endothelial growth factor (VEGF). On the other hand, the accompanying epigenetic events mostly involve methylation, acetylation and seemingly most relevant non-coding RNAs, an example being microRNA-200, which was proven to be crucial for the EMT–MET balance (Brabletz 2012). However, there is scepticism in the scientific community regarding these hypotheses, pointing to the fact that there is no convincing evidence for conversion of epithelial cells into mesenchymal cell lineages in vivo, proposing that the biological repertoire of malignant cells themselves is sufficient to account for their invasion without the need to invoke the radical change of the cell's identity (Tarin 2005).

15.2 Brain Tumors

15.2.1 Glioblastoma Molecular Classification

Glioma originate from glia (the Greek for 'glue'), the brain tissue that was traditionally viewed to functionally support neural cells. Glioma diagnosis has historically been based on examining the cellular morphology of the tumor to assess its presumed cellular origin (astrocytic, oligodendroglial, ependymal) and surrogate markers of tumor aggressiveness (necrosis, nuclear pleomorphism, mitosis) to determine the tumor grade (Ohgaki and Kleihues 2007). The major groups are astrocytoma (WHO grades I and II), anaplastic astrocytoma (WHO grade III) and glioblastoma multiforme (GBM; WHO grade IV) (WHO 2012). Survival of patients with GBM, the most aggressive glioma, although individually variable, has improved from an average of 10 months to 14 months after diagnosis in the last 5 years owing to improvements in standard care with an emphasis on targeted therapy (reviewed by Van Meir et al. 2010). GBM is thought to arise de novo (primary GBM) in about 95 % of cases or through the malignant transformation of lower-grade astrocytic and oligodendroglial tumors (secondary GBM). Over the

past decade, multiple genetic (transcriptomics, proteomics) and recently various epigenetic alterations have been associated with each type of GBM (reviewed by Bleeker et al. 2012). Currently, a few defined distinguishable histopathological differences exist that are associated with primary and secondary GBM prognosis and are useful in clinical practice. Several oncogenes (such as *EGFR* and *PFGFR*), tumor suppressor genes (such as *PTEN*, *TP53* and *RB1*) and other individual biological markers such as ECM molecules (e.g. tenascin C; Hirata et al. 2009), kinases (e.g. Wee1; Mir et al. 2010), proteases (e.g. urokinase plasminogen activator, cathepsin B; reviewed by Lah et al. 2006), metabolic enzymes (lactate dehydrogenase 1 mutations; Atai et al. 2011) and various stem cell markers, including CD133 (Singh et al. 2003; Strojnik et al. 2007; Zeppernick et al. 2008; Lathia et al. 2011), have been individually recognized to drive GBM progression and impact its prognosis. Still these individual clinicopathological and molecular data failed to correlate to therapeutic response in most patients.

Therefore, studies on the molecular features of glioma have recently come to the forefront with the advent of high-throughput microarrays and other molecular technologies. A molecular biology approach was initially attempted by Tso et al. (2006). They used large-scale expression analyses of glioma-associated genes to find some differences that distinguish between primary and secondary GBM and also to confirm the degree of heterogeneity within both groups. Moreover, secondary GBM was shown to primarily include glioma-associated genes related to mitotic cell cycle components, suggesting the loss of function in prominent proliferation regulators, whereas in primary GBM, genes typical of a stromal response were highlighted, suggesting the importance of extracellular signalling and possibly tumor–stroma interactions. Further, Tso et al. (2006) confirmed the mesenchymal properties in some of primary GBM tumors associated with stem-like properties of mesenchymal lineages, pointing to the question regarding the cellular origin of this GBM subset. At about the same time, Phillips et al. (2006) used a similar approach, but focused more on patient survival and revealed that one tumor class displaying neuronal lineage markers also displayed longer survival, whereas two other tumor classes enriched in neural stem cell (NSC) markers displayed equally short survival and exhibited additional markers of proliferation, angiogenesis and mesenchyme. On recurrence, these tumors frequently shifted towards the mesenchymal subclass. This work suggested that molecular classification of GBM may predict response to targeted therapies. Liu et al. (2006) and later others (Gunther et al. 2008; reviewed in Riddick and Fine 2011) postulated that gene expression profiles provide a transcriptome snapshot of a biological phenotype and offer the opportunity for quantitative evaluation of individual tumor biology. This may thus lead to the era of personalized diagnosis and prognosis and possibility of defining patient subgroups within a tumor class that would unequivocally respond to defined treatment. By understanding the biology of each group, one may better select a specific therapy tailored to the group and test it in a clinical trial (Chin and Gray 2008; Van Meir et al. 2010). Along these lines, The Cancer Genome Atlas (TCGA) consortium, sponsored by the National Institutes of Health, was built to understand GBM biology through

the integration of DNA copy number, methylation patterns and gene and microRNA expression data (Verhaak et al. 2010). This multicentric study integrated multidimensional genomic data to establish patterns of somatic mutations and DNA copy number to describe a robust gene-expression-based molecular classification of GBM into proneural, neural, classical and mesenchymal subtypes. Since temozolomide therapy and radiotherapy are commonly (postsurgically) used in GBM patients, a significant increase in survival was observed in the classical and mesenchymal subtypes, which are characterized by specific expression of *EGFR*/*NF1* compared with the proneural subtype, with characteristic aberration in *PDGFRA*/*IDH1*, indicating that additional therapies targeted to the two distinct signalling pathways would be more beneficial. Besides somatic mutations, chromosomal copy number alterations were put in the context of three distinct receptor tyrosine kinase (RTK) pathways, such as RTK–Ras–phosphoinositide 3-kinase (PI3K), retinoblastoma (Rb) and p53 signalling, all previously shown to be differentially deregulated in glioma (Van Meir et al. 2010; Verhaak et al. 2010; Riddick and Fine 2011). In an independent cohort of 91 tumors, 88 % of patients showed alterations in the RTK–Ras–PI3K pathway, 87 % showed alterations in the p53 pathway, and 78 % showed alterations in the Rb pathway. Overall, 74 % of patients showed changes in all three pathways. This study pointed to the fact that all subgroups commonly demonstrate the inactivation of the p53 and Rb tumor suppressors and activation of the RTKs by *EGFR*, *ERBB2*, *PDGR*, or *MET*. The TCGA has thus dramatically accelerated our understanding of the gene mutations and expression profiles of GBM in a multidimensional fashion, thereby defining the altered key signalling pathways that ought to be targeted for therapy. Similarly, Li et al. (2009) proposed the existence of two major groups of gliomas (oligodendroglioma-rich and glioblastoma-rich groups) separated into six hierarchically nested subtypes by identified classifiers when using internal and two external data sets of patient groups. Applying this classification system to the external glioma data sets allowed the identification of previously unrecognized prognostic groups within previously published data and within the TCGA GBM samples. Li et al. (2009) thus suggested that these biological pathways associated with the different glioma subtypes offer novel potential clues for tumor pathogenesis and therapeutic targets within each tumor subtype. As recently pointed out by Riddick and Fine (2011), only further integration of the gene expression, single-nucleotide polymorphism, proteomic, microRNA, and epigenomic data could fine-tune and increase the reliability and biological interpretability of the results. At present, this is still not ideal for prognosis and therapy response prediction, since the large statistical variability in the prediction of the response of the genetic fingerprint to therapy cannot be neglected. The validation studies by Marko et al. (2011) pointed to the role of technical factors in molecular classification inconsistencies in GBM, which besides their biological variability display abnormal data distribution. Also, non-linear relationships among genes may be responsible for classification errors. These findings may have important implications for basic GBM research and for clinical translation of expanded ‘omics’ databases.

15.2.2 Glioblastoma Invasion

The results from the studies described in the previous section certainly promote the development of novel therapeutic strategies targeting the most relevant biological makers to combat the resistance of GBM and GBM stem cells (see later). One of the treatment aims is to target the GBM invasiveness, which is the major characteristic of infiltrative astrocytic tumors. During malignant progression, the GBM borders become ill defined as the tumor zone progressively transitions into normal tissue. There, so-called guerrilla cells invade and infiltrate the normal tissue presumably by a mesenchymal type of movement, involving adhesion to the ECM, its degradation and migration (Friedl and Wolf 2003). High invasiveness is the major obstacle for successful GBM therapy, as the cells that migrate away from the growing tumor mass are hard to reach by surgery, radiation or local and systemic non-targeted drug delivery. As methods to track them in patients do not exist, it is unclear to what extent existing therapies reach and affect these cells. The key question that needs to be resolved prior to designing an anti-invasive therapy is to define the molecular repertoire of the migrating cells, which depends on the surrounding normal brain parenchyma and glioma-modified ECM. Both were shown to dictate the cell switch from the stationary to the migratory phenotype, by altering cellular morphology and/or by perturbing the 'cancer degradome' (Lopez-Otin and Matrisian 2007) by activating certain proteolytic cascades, e.g. the set of proteases, needed to modify the glioma matrix. Wolf and Friedl (2011) furthermore emphasized the importance of the ECM structure and composition when discussing whether or not pericellular proteolysis is required for cell migration, and claimed that migration can range from protease-driven movement to a completely non-proteolytic, non-destructive (ameboid) movement that depends on the deformability of the cells. The morphological flexibility of the cancer cell encounters the cell's ability to undergo EMT, as well as the transition from mesenchymal to ameboid phenotype, depending on the available tissue space (Friedl and Wolf 2003; Schmidt and Friedl 2010).

Therefore, in GBM the induced motility may also depend on EMT of GBM and/or GBM stem cells, although mesenchymal to ameboid transition would be more likely, owing to the special brain ECM composition and non-epithelial morphology of migrated glial cells. Typical dispersion routes in brain include white matter tracks and routes along the basal lamina of brain blood vessels, or in between the glia membrana limitans and the pia mater (Bellail et al. 2004). There the ECM is largely composed of the polysaccharide hyaluronan and proteoglycans, mostly as hyaluronic acid binding secreted chondroitin sulfate proteoglycans of the lectican family (aggrecan, brevican, neurocan and versican). Migrating tumor cells must thus navigate through ECM tissue structures of complex and varying physicochemical properties, including molecular composition, porosity, alignment and stiffness, by adopting strategies that involve deformation of the cell and engagement of matrix-degrading proteases (Schmidt and Friedl 2010). In GBM, the overexpression, translocation and activation of proteases is induced by integrins and RTKs, including epidermal growth factor receptor (EGFR) and

platelet-derived growth factor receptor (possibly fibroblast growth factor receptor), assisted by the Src family of non-receptor tyrosine kinases and G proteins of the Rho family of small GTPases. This way the neoplastic cells are guided via changed shape through the interstitial gaps and spaces in the tumor-surrounding matrix to find permissive paths for enhanced migration. The need for protease activation in GBM invasion may depend on a particular GBM subtype, as discussed above. Interestingly, differential transcription profiles of the invasive versus the stationary glioma cells in an in vitro model revealed a signature of 22 genes classifying GBM cell cultures and correlating well with their migration rate. These genes were also affected in 75 % of human GBM tumors (Demuth and Berens 2004), among which, surprisingly, no genes coding for proteolytic enzymes were found. The latter were contrarily confirmed to play an important role in GBM invasion (reviewed in Levicar et al. 2003; Rao 2003; Lah et al. 2006) in a number of in vitro and in vivo animal studies as well as in clinical studies. Likewise, our group focused on studying lysosomal cathepsins, which have been recognized as the initiators in the proteolytic cascade associated with pericellular proteolysis. Lysosomal cathepsins comprise mostly 13 cysteine cathepsins, of which cathepsin B seems to be an invasive marker of GBM (Strojnik et al. 2005; Colin et al. 2009; Gole et al. 2009) and other tumors (Withana et al. 2012). By means of an in vitro spheroid model study similar to that of Demuth et al. (2008), we confirmed the upregulation of the activity of cathepsins B, L and S, which did not result from an increase in messenger RNA levels in the invading GBM cells (Gole et al. 2009, 2012). The upregulation of the activity of cathepsins proved to be the result of post-transcriptional regulation, affecting the protein ratio between enzymes and their endogenous inhibitors, which is particularly relevant in the case of cathepsin B. In animal models, cathepsin B was demonstrated to be localized at the tumor cell surface, where it comes into contact with ECM proteins and may potentially activate a known proteolytic cascade, including urokinase plasminogen activator, plasminogen and metalloproteases, together resulting in enhanced cell invasion. Similarly, proteolysis was enhanced in co-cultures with stromal cells, as demonstrated recently by Sameni et al. (2012) in other tumor systems. Even though the concepts described above call for therapeutic targeting of proteases to prevent migration and invasion-associated tissue destruction, the full characterization of an invasive cell subpopulation in GBM needs to be completed, since it may be that these invasive cells may have stem cell characteristics and/or that CSCs may undergo EMT (Borovski et al. 2009, 2011) as discussed later.

15.2.3 Cancer-Associated Stem Cells: Glioma-Propagating Stem Cells

A theory that cancer originates from stem cells was first postulated in the nineteenth century from observations of the histological similarities between developing fetal cells and teratocarcinoma. Further comparison of embryonic and

cancer development led scientists to propose that cancers originate from a small population of embryonic cells 'forgotten' in the tissues of the adult organism. The modern concept of cancer originating from so-called tissue stem cells transforming into CSCs, however, appeared at the turn of this century (Reya et al. 2001). It postulates a tumor as an abnormal organ, which to a certain degree follows the dedifferentiation programme encoded within the CSCs. Also, similar to normal stem cells, CSCs have an unlimited ability to divide symmetrically and asymmetrically, resulting in progenitor cells, losing the differentiation potential during the subsequent asymmetric divisions, but increasing proliferation via symmetric divisions. A monoclonal origin of tumors and the stochastic nature of carcinogenesis was first challenged by the simple hierarchical model (Vescovi et al. 2006) and was followed by variations of mixed models (Huse and Holland 2010) that predict the existence of various clones of so-called tumor-propagating cells (TPCs), also termed true CSCs. The term 'tumor-propagating cells' (TPCs) is used here to clearly distinguish these cells from tumor-initiating cells (TICs), which are the first to undergo transformation/mutation that cannot be repaired and is transferred to daughter cells, and the TPC-CSCs which can regrow the original tumor in an appropriate microenvironment and are also highly resistant to therapy. Current CSC theory also hypothesizes that heterogeneity within tumors is not a mere consequence of random mutations and clonal evolution, but results from an intrinsic hierarchy of cells, with the top-situated CSCs (Wicha et al. 2006; Tysnes and Bjerkvig 2007; Prestegarden and Enger 2010; Van Meir et al. 2010), as illustrated in Fig. 15.1. Furthermore, the mixed combined model proposes plasticity of CSCs, meaning that by dedifferentiation an even more aggressive population of CSCs may evolve through acquisition of additional mutations or epigenetic modifications. This secondary aggressive CSCs may become dominant and drive tumor formation or may simply coexist with other phenotypes of CSCs (Visvader and Lindeman 2008). In their review, Visvader and Lindeman (2008) discussed the localization of CSCs within the primary tumor, metastases and xenotransplants in animal models, emphasizing the importance of an appropriate 'niche formation' for their accommodation. The niche is composed of stromal elements, including fibroblasts, endothelial cells and immunocompetent cells (Bjerkvig et al. 2009; Borovski et al. 2011). All these 'niche' cells can also differentiate in situ from the infiltrated normal bone-marrow-derived stem/progenitor cells as a result of cellular cross talk with tumor cells (Birnbaum et al. 2007; Ricci-Vitiani et al. 2008) (see Sect. 15.1.3). Underpinning this statement, Ricci-Vitiani et al. (2010) showed that a portion of endothelial cells in GBM actually carry the same genetic mutations as tumor cells, and that in GBM xenografts the endothelial lining of the vessels was primarily composed of the donor (human) cell's of origin. Wang et al. (2010) likewise confirmed a fraction of endothelial cells within the tumor trace to a special subset of $CD133^{+}$ GBM stem cells, which indeed have the ability to differentiate into endothelial progenitors. Their transition to endothelial progenitors seem to be VEGF-dependent, whereas further maturation to endothelial cells relies on the effective Notch-1 signalling. High relevance of the niche was demonstrated by Quintana et al. (2010), who showed that in highly immunosuppressed

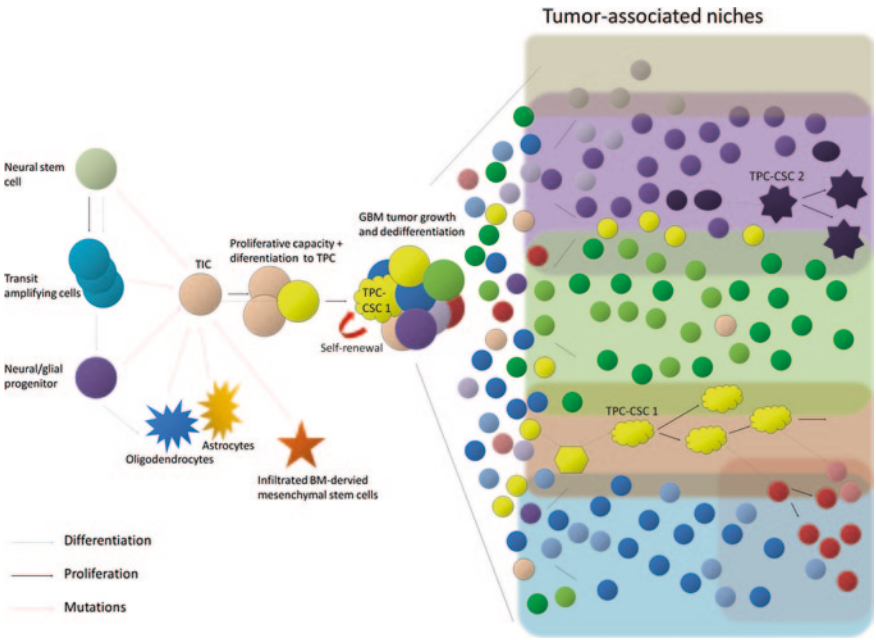

Fig. 15.1 Modern cancer stem cell concept. Suggested lineage relations for the maturation and production of tumor-propagating cells (*TPC*)—cancer stem cells in glioblastoma multiforme (*GBM*)—and a possible explanation of cellular tumor progression are shown, GBM may develop from various normal cell lines present in the brain, such as neural stem cells, their progenitors, mature differentiated cells and possibly infiltrating mesenchymal stem cells (MSCs) that undergo genetic alterations (mutations), which leads to a population of tumor-initiating cells (*TIC*). TICs may proliferate faster than normal cells and they also accumulate further genetic and epigenetic changes to generate the true tumor stem cells, the TPCs, capable of propagating the whole tumor, and these are also called cancer stem cells (*CSC*). These are highly resistant to DNA damage and dedifferentiate to produce various tumor progenitor cells within the tumor mass, which are responsible for the clonal development of numerous functional tumor cells, according to the stochastic model of cancer development. According to the hierarchical model, only TPC-CSC 1 are tumorigenic in a sense that they are programmed to produce the same type of tumor, e.g. the formation of a certain genetic subtype of GBM. However, this subtype will accommodate specific mutations allowed by and reflecting the traits of the cell of origin. During further development of multiple tumor clones, there may be a random formation of novel tumor cell clones that develop stem-cell-like properties, including self-renewal and multipotency (TPC-CSC 2 clone). Whereas most tumor clones will only survive in specific well-adapted niches, the TPC-CSCs clones are more flexible and will adapt to less optimal conditions, such as hypoxia, where their divisions follow a hierarchical programme of development. Depending on the niche/microenvironment, these cells may alter the repertoire of stem cell markers owing to reversible epigenetic changes dictating their behaviour with respect to migration and proliferation. On the basis of the proposed concepts described in the text, the scheme was adopted from Fig. 1 in Bjerkvig et al. (2009) and Figs. 1 and 3 in Van Meir et al. (2010). *BM* bone marrow

NOD/SCID IL2Rγ null mice, which lack the natural killer activity of the wild-type NOD/SCID mice, tumors could be successfully initiated from a single melanoma cell, on which—surprisingly—no stem cell surface marker enrichments had

any influence. It may therefore be hypothesized that cancer is a stem cell disease, as claimed by many (Wicha et al. 2006; Tysnes and Bjerkvig 2007; Van Meir et al. 2010), and sceptically discussed by others (Bjerkvig et al. 2009), where the work of Quintana et al. (2010) could possibly be only melanoma-related.

It has been hypothesized that different subsets of GBM stem cells may underlie the growth of all six genetically defined GBM subtypes (Van Meir et al. 2010; Verhaak et al. 2010; Zong et al. 2012). Overall, it is accepted that glioma originate from the malignization of an astrocytic lineage (Ohgaki and Kleihues 2007). However, during normal central nervous system differentiation, NSCs undergo amplification to produce transit-amplifying progenitor cells (type C cells), which then differentiate into neural/glial progenitor cells. These progenitor cells retain the capacity to produce progeny along either neural or glial lineages (oligodendrocytes and/or astrocytes), but never both. The actual origin of GBM stem cells has thus still been not clarified, as the initial proposal that they arise from mutated normal NSCs has been challenged by the hypotheses that all NSC progenitors as well as differentiated astrocytes on dedifferentiation can undergo transformation by various mechanisms of cell fusion, induced transdifferentiation and phagocytosis of apoptotic bodies (Bjerkvig et al. 2005). Mutations generating GBM tumors could thus occur at all levels within these lineages and produce subtypes of glioma TICs.

Also infiltrating BM-MSCs could contribute to malignization of existing tumor/transformed cells by promoting the cell's acquisition of stemness-like properties (Houghton et al. 2007; Shiras et al. 2007; Tysnes and Bjerkvig 2007; Visvader and Lindeman 2008). We believe that all currently available data actually support the working hypothesis proposed by Bjerkvig et al. (2009), which combines the observed GBM stem cell plasticity (mixed model) and different TIC lineage origins with the niche interactions, predicting the creation of stable TPC-CSC phenotype(s) within a tumor during tumor progression (Piccirillo et al. 2009) (Fig. 15.1). A fusion of a (genetically damaged) differentiated tissue cell with a tissue stem cell or a recruited stem cell from bone marrow is also not excluded. These processes result in increasing cellular heterogeneity and hierarchy within the tumor originating from various CSC clones (Chen et al. 2010). It is now generally believed that the TCGA classification of GBM subtypes (described in Sect. 15.2.1), based on transcriptomic fingerprints, indeed results from distinct glioblastoma TPC-CSC types (Hadjipanayis and Van Meir 2009; Van Meir et al. 2010).

15.2.3.1 Glioblastoma Cancer Stem Cell Molecular Markers and Their Clinical Impact

According to the hypotheses discussed in the previous section, the term 'cancer stem cell' (CSC) is therefore just a working term since no defined molecular genotype of such cells exists, but instead the term describes a set of tumor-associated cells which have the required CSC characteristics. Nevertheless, how to study these cells in the in vitro and in vivo models remains a challenge. The sphere formation assay was adapted to enrich sphere with a putative GBM stem

cell population similar to normal NSCs (Singh et al. 2003; Yuan et al. 2004). This way GBM stem cell enriched neurospheres grown from primary brain tumors were shown to possess an increased cancer-initiating ability of 1,000–5,000 cells to reinitiate the tumor when xenotransplanted into the brains of NOD/SCID mice in vivo, compared with the initial primary tumor population (Potet et al. 2011). The stemness of GBM stem cells is conditioned by several old and new stem cell markers, such as SOX-3, MSH-1, CD15, integrin 6, A2B5 and L1CAM (Dell'Albani 2008; Lathia et al. 2011) and the Hedgehog, Notch and BM1 signalling pathways (reviewed by Zong et al. 2012).

Of these, the GBM stem cell isolation and identification assays have so far focused mostly on a single cell surface epitope, the plasma membrane associated protein CD133/prominin-1, defining also stemness of primitive cells from epithelial, endothelial, haematopoietic and neural tissues (Potet et al. 2011), including brain (reviewed in Hadjipanayis and Van Meir 2009; Van Meir et al. 2010). Several studies confirmed variable expression of CD133 among high-grade glioma samples, and $CD133^-$ cell populations from GBM were also shown to have tumor-initiating potential, giving rise to $CD133^+$ tumors (Sakariassen et al. 2006; Beier et al. 2007; Joo et al. 2008; Wang et al. 2008). The functional role of this marker in tumor progression still needs to be resolved, especially as its content in GBM ranges from 2 to 30 %. In contrast, the abundance of nestin, another CSC marker, can be as high as 80–90 % in most of the GBM samples examined (Strojnik et al. 2005). Yet, both CD133 and nestin, along with SOX-2 and MSI-1, were shown to increase in expression with malignancy and had bad predictive value for patient survival (Sugawara et al. 2002; Strojnik et al. 2007; Ma et al. 2008a, b; Rebetz et al. 2008; Zeppernick et al. 2008; Ardebili et al. 2011). Some rare (0.02–0.5 %) $CD133^+$ cell populations were found to exist also in established U87MG and U373MG glioma cell lines, which could both be additionally enriched for $CD133^+$ and nestin populations displaying multipotent characteristics of CSCs by the use of NSC media (Yu et al. 2008). This indicates high plasticity of the stem cell phenotype, dependent on the environmental conditions in vitro and in vivo.

The concept of CSCs has a significant clinical impact on diagnosis and prognosis. The final goal of the tremendous efforts put into investigation of GBM stem cells is to efficiently target these subpopulation of cells, which are most resistant to radiotherapy and chemotherapy, owing to their higher expression of drug-resistance proteins such as ATP-binding cassette half-transporters (ABCG2 and ABCG5), multidrug resistance protein 1 (MDR1) transporters (Donnenberg and Donnenberg 2005), apoptotic resistance (Bao et al. 2006) and highly active DNA repair (Dean et al. 2005; Huse and Holland 2010). As discussed above, during tumorigenesis, the characteristics of CSCs are prone to change; therefore, a distinct CSC could represent a 'moving target'. In addition, Van Meir et al. (2010) claim that not only GBM stem cells but also their progenitors should be targeted simultaneously. In practice, combinatorial treatments involving both cytotoxic and targeted therapies will probably be required to ablate all cancer and stromal cells composing the GBM stem cell niches. With respect to targeting GBM stem cells, the stemness- and proliferation-related signalling pathways (PI3K OLIG2, SHH,

Notch and Wnt), which were found to be essential for the development and regulation of normal and GBM stem cells, could be considered as candidate targets (Ma et al. 2008a). Alternatively, inducing apoptosis, senescence and the differentiation of GBM stem cells may be a therapeutic mechanism for targeting GBM (Hadjipanayis and Van Meir 2009).

15.2.3.2 Glioblastoma Stem Cell Niches

With respect to the GBM stem cell niches, both the perivascular and the hypoxic areas have been proposed to best accommodate GBM stem cells (Heddleston et al. 2009). This apparent contradictory notion of a normoxyc/hypoxic requirement can only be explained by the high plasticity of GBM stem cells and/or by their reversible phenotype transitions, reminiscent of the reversible EMT process. Regarding that, Bjerkvig et al. (2009) postulated a hypothesis on hypoxia keeping CSC-like cells in a hypomethylated state and enhancing tumor stemness by increasing the invasive and tumorigenic ability, as shown by the tumor side population (Das et al. 2008) residing in the hypoxic niches within GBM. CSC-like cells show an upregulation of Akt kinase, which is associated with increased glycolysis, antiapoptotic activity and cell migration, similarly as in normal NSCs. Through asymmetric cell divisions, GBM stem cells could propagate to the progenitor cell clones with higher oxygen requirement and angiogenesis dependence for further growth and survival. This was confirmed in the NOD/SCID mice model, where both an angiogenesis-independent and an angiogenesis-dependent stem cell phenotype in vivo were confirmed (Sakariassen et al. 2006). Antiangiogenic therapies may well affect oxygen-dependent GBM stem cells, which rely on oxidative respiration for their growth, whereas GBM stem cells that do not depend on the oxygen level can survive under these conditions (the so-called Warburg effect) and invade the surrounding tissue (Miletic et al. 2007). Experiments demonstrated that nestin^{+}/CD133^{+} cancer cells are also located in the perivascular regions of GBM, where the endothelial cells maintain these cells in the self-renewing and undifferentiated state. From the perivascular niche, these CD133^{+} cells may be eliminated on administration of antiangiogenic therapies (Calabrese et al. 2007). These findings somehow contradict clinical observations of inefficient antiangiogenic therapies using bevacizumab or cediranib when survival was taken as the end point. The failure of antiangiogenic therapy implies the subsequent regrowth of the tumor, from which it can be speculated that in human GBM a CD133^{-} cell subpopulation with stem-like properties may exist and is responsible for tumor relapse after antiangiogenic therapy. As hypoxia was shown to regulate the behaviour of CSCs within tumors (Seidel et al. 2010), CSCs differentially expressing various hypoxia-inducible factors (HIF), including the HIF2α subtype, were confirmed to reside also in GBM (Li et al. 2009). Further, it was shown that knockdown of HIF1α inhibits both CSCs and non-CSCs, whereas knockdown of HIF2α selectively decreases only survival of CSCs in vitro and impaired tumor formation in vivo. In addition, Hashimoto et al. (2011) reported on the expression of stem cell signature

genes, including CD133, which are upregulated in cultured pancreatic cancer cells under hypoxic conditions, causing the overexpression of HIF1α and increase of their metastatic and tumorigenic ability.

In gliomas, Donovan and Pilkington (2012) demonstrated that CD133 expression coincided with the hypoxic regions within human GBM tumors and cells highly expressing the GBM stem cell markers nestin, OLIG2, MSH-1 and SOX-2, the levels of which all increase also in vitro with decreasing oxygen tension. They hypothesized that hypoxia and the underlying mitochondria functionality, both triggering HIF promoters, are critically responsible for complex regulation of CD133 in GBM stem cells. As in other tumors, HIF2α is highly expressed in GBM $CD133^+$ cells, whereas HIF1α is expressed in $CD133^-$ cells. Thus, it was proposed that the microenvironment may control the GBM stem cell CD133-dependent biological behaviour, with HIF2α stabilizing the highly proliferative nature of $CD133^+$ cells via the Notch-1 signalling pathway, whereas HIF1α may contribute to the invasive and angiogenic phenotype of $CD133^-$ cells via VEGF (Donovan and Pilkington 2012). The latter may also be associated with an increased migratory and invasive phenotype of GBM stem-cell-like cells proceeding via a kind of EMT process (Visvader and Lindeman 2008; Borovski et al. 2011; Schubert and Brabletz 2011; Brabletz 2012), as already discussed in Sect. 15.1.3. For example, in pancreatic tumor the concept of a metastatic CSC was proved by Hermann et al. (2007), who defined a distinct subset of $CD133^+CXCR4^+$ cells that on binding the chemokine CXCl12/SDF1 localized to the invasive edge of pancreatic carcinomas and exhibited significantly stronger migratory activity in vitro than $CD133^+CXCR4^-$ cells despite both subsets undergoing similar tumorigenesis. Significantly, only $CD133^+CXCR4^+$ cells demonstrated in vivo metastasis to the liver. Presumably also GBM-associated $CD133^-$ cells on reaching the secondary site could seed in normal brain parenchyma, where they could become stationary by an MET-like process and grow a new GBM lesion. A model was thus proposed in which $CD133^+$ cells constitute a non-invading GBM stem cell population with the potential to switch reversibly between the stationary and the invasive $CD133^-$ phenotype, associated with gained and lost activity of migratory proteins. During that process, a distinct set of cancer degradome proteases, associated with invasion, might also be upregulated. Similarly to Donovan et al. (2012), we found higher invasion of GBM $CD133^-$ cells under normoxic conditions (Ardebili et al. 2011). Also, higher invasion levels accompanied by a distinct pattern of collagen type I degradation were found in normal NSCs compared with $CD133^+$ spheroids isolated from primary GBM tumors, whereas the higher proliferative ability of $CD133^+$ spheroids in collagen-embedded cultures was demonstrated, as expected. Some inverse correlation was confirmed between CD133 expression and the lysosomal cathepsin B—the invasion marker in clinical evaluation of primary GBM tumors, in which lower cathepsin B activity was observed in $CD133^+$ cell fractions. These results suggest that GBM stem cells are not as invasive as their progenitors that lose CD133 and acquire migratory properties by the activation of invasiveness-related proteolytic enzymes. We believe that as the tumor progresses, genetic and epigenetic

mechanisms in GBM stem cells that drive tumorigenesis may result in the emergence of a self-renewing invasive GBM stem cell that expresses different cell surface markers, yet lacks CD133 expression.

15.3 Mesenchymal Stem Cells as Part of the Tumor Microenvironment and Therapeutic Vectors

15.3.1 Characteristics of Mesenchymal Stem Cells

Stem cells associated with tumors are also infiltrating haematopoietic stem cells, endothelial progenitor cells and MSCs. They are highly migratory and exhibit a tropism towards tumors, mediated by various cytokines. Their role and differentiation pathways in the 'tumor tissue' niches are the subject of intensive investigations.

In particular, human MSCs have been extensively studied, to understand not only their basic biology but also their intrinsic properties, such as self-renewal, differentiation, their pathotropic migratory properties associated with homing and their immunomodulatory potency, which all point to the potential of MSCs to be used as a promising cell/drug delivery vehicle in cell-based therapies. MSC-based strategies have already been proposed for treating neurodegenerative diseases, heart failures and other organ injuries as well as incurable cancers. Their counterpart, pluripotent human embryonic stem cells, which are an in vitro equivalent of pluripotent cells residing in the inner cell mass of the blastocyst-stage embryo, at this point face critical ethical issues that are stalling their research. Alternatively, induced pluripotent stem cells amenable for genetic manipulation from various differentiated tissue cells have been derived. Unfortunately, access to them is rather limited and currently their production is too expensive for clinical application. For this reason, isolation and characterization of multipotent MSCs as putative adult analogues of embryonic stem cells is of such importance.

MSCs, initially also called tissue or adult stem cells, are more or less present in all tissues and have a vast differentiation potential (Dominici et al. 2006). They are defined by a panel of phenotype characteristics and molecular markers (Dominici et al. 2009; Motaln et al. 2010). The MSCs must pass three minimal criteria: (1) the ability to adhere to plastic, (2) the presence of more than 95 % expression of MSC-specific antigen markers and more than 95 % absence of expression of haematopoietic/endothelial markers and (3) the potential to differentiate into osteoblasts, adipocytes and chondroblasts in vitro under defined conditions. Moreover, MSCs phenotypic differences were observed regarding their tissue origin; therefore, a detailed description of cell markers and their behaviour, defined by biological and functional assays, could in future allow us to decide precisely which cell source to use for a certain therapy in a certain individual. This also leads us to the conclusion that the pool of MSCs within an organism actually consists of a heterogeneous MSC population.

In tissues, MSCs reside in the so-called tissue stem cell niches, which comprise stromal cells, such as fibroblasts and particular ECM components. Although MSCs are relatively easy to isolate from various adult tissues, the numbers of MSCs inhabiting niches in different tissues may differ greatly. The highest efficiency of MSC isolation has been reported for bone marrow aspirate, fat tissue and umbilical cord. In contrast, MSC yields from fresh umbilical cord blood range from 30 to 65 % and are even lower in cryopreserved umbilical cord blood samples, where the yield is assumed to be below 20 % (Motaln et al. 2010). Also, amniotic membrane and umbilical cord are becoming attractive sources of MSCs, since these fetal tissues are discarded without any ethical issues. In our hands, umbilical cord Wharton's jelly offered the highest yields (unpublished data) of MSCs, free of vascular haematopoietic cell contamination as observed during MSC isolation from umbilical cord blood (Alviano et al. 2007). Umbilical cord MSCs are also characterized by the highest proliferation rate, presumably owing to their high telomerase activity (Bentzon et al. 2005). Although umbilical cord blood MSCs could provide a young and genetically undamaged cell pool, in contrast to adult tissue MSCs, which often display reduced 'fitness' and genetic damage, their acquisition from fetal tissues is time-limited. In contrast, adipose tissue MSCs (AT-MSCs) or BM-MSCs have the advantage of being easily harvested and autografted at any age from and into any patient. The advantage of using autologous stem cells such as MSCs in cell transplantations is their immunological compatibility, which has been shown to have a profound effect on transplanted cell survival. Yet their use may be limited by low absolute amounts of MSCs isolated from bone marrow, which necessitates their in vitro expansion prior to clinical use.

Although BM-MSCs possess the highest lineage plasticity (reviewed in Shah 2012), their application relies on a quite invasive isolation procedure. Therefore, more easily available AT-MSCs have been receiving increased attention recently. Protocols for isolation of MSCs from different tissues have already been improved, as have their culture, storage and transport conditions (Gordon et al. 2001; Rebelatto et al. 2008). Some novel technological approaches are in progress (Teo and Vallier 2010) and have been extensively reviewed by Loebinger and Janes (2010) and Grisendi et al. (2010).

Furthermore, the growth rate of MSCs in vitro shows an origin dependence. It seems that heterogeneity applies not only to different tissue sources (Kogler et al. 2004), but also arises between individuals owing to biological variability, since different clones were derived from tissue samples of different individuals. Also variations in terms of their life span and stem cell plasticity have been observed (Motaln et al. 2010). Senescence-associated growth arrest occurring during in vitro culture highly limits the extent of MSC proliferation. The expansion potential of MSCs is highly affected by in vitro culture conditions, such as the presence or absence of different growth factors (Sotiropoulou et al. 2006). For example, growth factors such as transforming growth factor (TGF)-β_2 gradually decrease MSC proliferation and by the same token MSC proliferation ceases during prolonged in vitro culture, owing to increased endogenous expression of TGF-β_1/TGF-β_2-R1 and SMAO3 (Sawada et al. 2006). In constrast, under hypoxic

conditions, as found in many tumors in vivo, MSCs continue to proliferate, displaying 30-fold higher expansion rates without the loss of their multilineage differentiation (Grayson et al. 2007). This could be utilized when treating tumors which exhibit chronic states of hypoxia, well accommodating CSCs, and where even low numbers of MSCs are present and may display a beneficial effect owing to their higher proliferative activity. Yet, addressing differences accompanying MSC growth under hypoxic conditions may also lead to MSC-like tumor stem cell derivation (Ricci-Vitiani et al. 2008) which in some tissues may even cause cancer development and persistence.

15.3.2 Mesenchymal Stem Cells in the Tumor Microenvironment

Tumor heterogeneity is related to two types of cells composing the growing tumor mass. First, it is related to the heterogeneity of malignant cells (Bonavia et al. 2011), reflecting the evolution of the tumor from TICs and tumor-propagating CSCs during tumor development and progression. The second cell type addresses the so-called stromal cells, which comprise the parenchymal tissue cells, surrounding and/or migrating into the growing tumor mass, and the haematopoietic/immune system related cells, infiltrating the newly formed tumor tissue via more or less abundant neoangiogenic vasculature. The latter, by themselves, can either originate by differentiation from infiltrated endothelial progenitors translocated to the tumor from bone marrow or burst out from existing surrounding blood vessels and lymphatic channels (lymphangiogenesis). Complex intercellular interactions between these non-malignant cells and tumor cells gradually change the tumor-associated stroma. Their paracrine interactions involve cytokines, growth factors with their receptors and tissue remodelling proteases, which were all initially supposed to mediate the spread of the tumor (Mueller and Fusenig 2004). On the other hand, more direct cell–cell interactions via gap junctions and exosomes undoubtedly play a role in direct cellular cross talk and are now being widely studied (Théry 2011). The tumor parenchyma comprising stromal cells is considered to be a new therapeutic target, since it is less resistant to cancer treatment.

MSCs are recognized as a part of the tumor stroma, where they are either activated from the endogenous tissue niches or released form bone marrow/adipose tissue and infiltrate into the tumor via blood circulation. Pluripotent MSCs and their progenitors in the peripheral blood have been extensively studied (Cesselli et al. 2009) and have been found to be highly migratory on TNF-α stimulation (Corallini et al. 2010). Tropism to tumors, as has been confirmed in various studies, is followed by interactions of MSCs with the tumor microenvironment. The latter is still not well understood, yet the process is strongly dependent on the tumor type, hypoxic condition and other stromal elements, including the ECM. Within the tumor, MSCs have been suggested to differentiate into tumor-associated fibroblasts (Grisendi et al. 2011), which have been shown to be

tumor-type-specific, depending on the histological characteristics, organ localization and progression phase of the tumor (Dominici et al. 2009). The generation of tumor-associated fibroblasts may involve either differentiation or transdifferentiation processes, where one of the key steps in the ontogeny of tumor-associated fibroblasts is proposed to also be an EMT (Thiery 2002; Kalluri and Weinberg 2009), a process similar to an endothelial to mesenchymal transition of the infiltrating MSCs (Potenta et al. 2008). Via this transition process, stationary CSCs undergo acquisition of a metastatic phenotype (Borovski et al. 2009, 2011). Also hypoxia regulators—HIF-1 and HIF-2α—were demonstrated to mediate the EMT (Giannoni et al. 2011) on culture oxygen deprivation, relevant to the intratumor environment. Alternatively, the role of molecules secreted by stromal and tumor cells that could possibly affect the phenotype of infiltrating MSCs should also be considered (reviewed in Kucerova et al. 2010; Motaln et al. 2010) since this was shown for GBM-associated macrophages (Zhai et al. 2011) and endothelial cells (Kenig et al. 2010).

In glioblastoma, it is thus believed that MSCs of bone marrow and/or peripheral blood origin enter the blood–brain barrier (Komatsu et al. 2010; Pati et al. 2011) and become GBM-associated MSCs. MSCs most likely enter the progressed GBM via extensive vasculature or infiltrate from the entire brain itself (Schichor et al. 2006; Kim et al. 2010). Even though GBM-associated MSCs have not been confirmed in tumors, attempts to isolate and characterize them are in progress. To mimic such MSC/tumor cell interaction, we studied GBM (U87MG) cells and BM-MSCs in direct co-cultures in vitro (Fig. 15.2). We showed that the MSCs intensively interact with glioma cells via the functional as well as structural syncytium that is formed, which alters the migratory potential and proliferative behaviour of both MSCs and GBM cells (Schichor et al. 2012). Further, we demonstrated that glioma cells as well as MSCs differentially express connexins during their interaction via gap-junction coupling. These complex cellular interactions lead to enhanced migration and altered proliferation of both cell types in vitro. The phenomenon described provides new insight into the complexity of interaction patterns between tumor cells and stromal cells. Moreover, structural syncytium formation with MSCs and GBM cells has gained additional attention as the potential of glioma cells to transdifferentiate into lineage-restricted cells of cardiogenic or neurogenic tissues was altered. In fact, there is growing evidence that fusion of MSCs with host tissue cells is an alternative mechanism of transdifferentiation (Kamijo et al. 2006). In summary, these results show that both cell-fusion-mediated and gap-junction-mediated cross talk are present in MSC/GBM cell direct co-cultures in vitro. These events should be considered as beneficial for accelerated drug delivery in MSC-based tumor therapies.

On the other hand, unwanted effects should be considered as well, and the risks of cell transplantation itself should not be underestimated. In this regard, Snyder (2011) Snyder has already thoroughly discussed the current drawbacks of MSC transplantations, which are due to the observed MSC cell masses appearing in the neoplastic, inflammatory brain lesions after transplantation. In an attempt to reveal the molecular mechanisms underlying the observed phenomena, we have

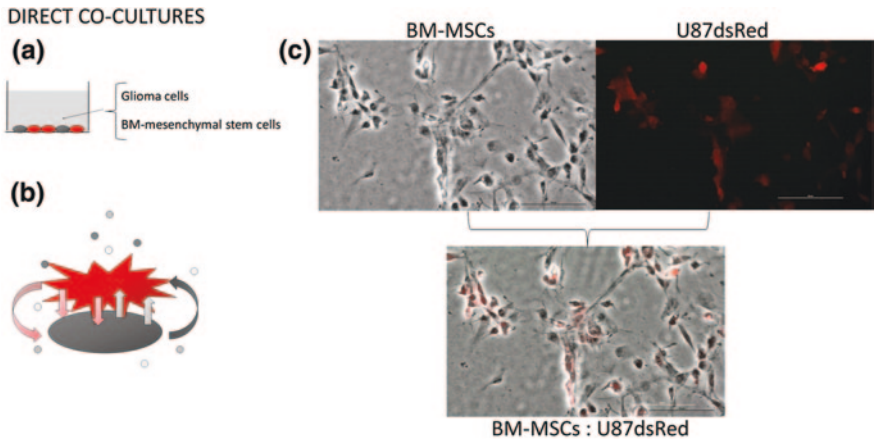

Fig. 15.2 Mesenchymal stem cells (MSCs) and glioblastoma cells in direct co-cultures in vitro illustrating the intratumoral communication between the U87MG GBM cell line and stromal BM-derived MSCs (*BM-MSCs*). **a** Cells were cultured in vitro in direct co-cultures. **b** In direct co-cultures, cells can directly influence each other by contact-dependent and feedback-loop-dependent signalling. Direct co-culture best resembles the situation of tumors in vivo. **c** U87MG GBM cell line and BM-MSCs in direct co-culture (magnification ×200). To better visualize the cells, U87MG cells were transformed by dsRed (*U87dsRed*), whereas areas not in *red* in the merged image represent BM-MSCs only. It is clear that some of these cells are in close contact, possibly forming the functional syncyitium, as we described in detail (Schichor et al. 2012). These merged clusters were subjected to transcriptomic analysis of joint messenger RNA, where by the mathematical deconvolution algorithm of monocultures and indirect cell cultures, we obtained the set of specific genes related to significantly altered expression of the genes owing to direct cellular interactions. (**c** Provided by Mateja Obrez)

used a MSC/GBM cell co-culture model and looked at the transcriptome of the GBM cell–MSC complex using the U87MG cell line and commercially available BM-MSCs (Lonza Bioscience, Walkersville, BD, USA; human MSCs 6F4085). The gene expression analysis of microarray data on direct co-cultures, indirect co-cultures and each of the cell monocultures was done using deconvolution—a mathematical procedure for separating the functions coming from mixed samples and thus detecting their differences. Deconvolution resulted in the finding of several interesting genes that are upregulated only when cells were in direct contact (Motaln et al. 2012). For example, bradykinin receptor (*BDKRB1*) gene expression appeared increased in direct co-cultures of U87MG cells and BM-MSC cells. Bradykinin regulates signalling of bradykinin receptors 1 and 2 (BDKRB1 and BDKRB2, respectively), differently triggering receptor-induced calcium response and neuron desensitization (Martins et al. 2008). Bradykinin signalling is also involved in neuronal differentiation and apoptosis (Martins et al. 2012; Montiel-Eulefi et al. 2012), as *BDKRB2* was reported to be highly upregulated in glioblastoma. Study of these pathway and other altered genes in GBM cell/MSC direct co-cultures, which is in progress, shall lead us to better understand the observed phenotypic changes emerging when the heterocellular complex is formed.

15.3.3 Mesenchymal Stem Cells as Therapeutic Vectors in Cancer

Over the last decade, advances in the understanding of stem cell biology have made cell therapy as a potential clinical reality very promising. Cell therapeutics may perform far better than current treatments based on recombinant proteins and chemical compounds only. Even though all these have proved effective as anti-tumor agents in experimental conditions and some even in clinical trials, several failed to perform well in therapy owing to their inefficiency to specifically target primary tumor cells, metastasis and/or tumor CSCs. In many cases the treatment also failed because of the adaptability of tumor cells, resulting in adverse effects, for example increased metastatic potential after administration of metalloprotease inhibitors (Kruger et al. 2001; reviewed in Lopez-Otin and Matrisian 2007) and administration of antiangiogenic agents (Miletic et al. 2007; Bjerkvig et al. 2009).

Cancer therapies based on cellular vehicles therefore seem to be most promising. As already mentioned, human embryonic stem cells and induced pluripotent stem cells are less suitable for tumor treatment owing to their high in vivo transformation/tumor (teratoma) forming potential (De Miguel et al. 2010). Thus, research and development has focused on novel stem cell therapeutics, which are also the subject of this chapter. BM-MSCs and AT-MSCs are the most attractive candidates for cancer therapy as they can be easily isolated and propagated to sufficient numbers in vitro. They can be genetically manipulated ex vivo and autologously transplanted into patients, this way overcoming immune rejection.

In cancer therapy development, the safety of the grafted progenitors is the major concern in clinical settings and was discussed by Momin et al. (2010). The potential of malignant transformation in vitro, after the cells have reached senescence, was indeed reported by some authors (Rubio et al. 2008) and disproved by others (Bernardo et al. 2007). Finally, this hypothesis was rejected on the basis of an international multiclonal study (Torsvik et al. 2010) which pointed to the critical artefacts occurring during in vitro propagation of MSCs. Importantly, non-immortalized adult stem cells do not confer the same danger as immortalized adult stem cells and may therefore be used without posing a risk to the patient (Shah 2012). Still, the issue is not completely resolved, particularly when addressing other unpredicted situations in vivo. In the complex tumor microenvironment though, cellular fusion and transdifferentiation upon MSC treatment may lead to increased tissue malignization as recently discussed (Schichor et al. 2012).

At present it is generally agreed that homing potential, i.e. the ability of bone-marrow-derived and blood-circulating MSCs to actively migrate towards the tumor in response to chemokines produced by the tumor mass, does exist (Ciavarella et al. 2011; Grisendi et al. 2011). For this reason, the exogenously delivered MSCs also localize preferentially to the tumor, this way proving applicable for clinical translation as a cellular therapy and overcoming the necessity for a whole bone marrow transplant (Loebinger and Janes 2010). Furthermore, irradiation promotes migration of MSCs towards the tumor and increases the apoptotic potential of MSCs with

TNF-related apoptosis-inducing ligand (TRAIL) in its secretable form (sTRAIL) (Francois et al. 2006; Kim et al. 2010). Altogether these phenomena offer new exploitation strategies for MSC treatment of 'hard to reach and remove' tumors. Although specific tropism to the tumors and incorporation of MSCs into malignant tissue have been demonstrated in various preclinical models, an increasing number of studies are reporting divergent tumor environment response to exogenously delivered MSCs. Data analyses are pointing to a bimodal action of rat and mouse BM-MSCs on in vitro tumor cell lines that is seemingly dependent on the tumor type. For example, Kucerova et al. (2010) demonstrated an MSC-mediated protective effect in melanoma A375 cells under nutrient-limiting and hostile environmental conditions after allowing for cross talk between neoplastic and non-malignant cells. In contrast, a tumor-inhibiting effect was observed in 8MGBA glioblastoma cells. In addition, Zhang et al. (2010) reported on the tumor-growth-promoting effect of MSCs in breast, lung and prostate cancer, whereas Khakoo et al. (2006) confirmed the antitumoral effects of MSCs in Kaposi sarcoma. Likewise, Lu et al. (2008) demonstrated the growth-inhibitory effect of BM-MSCs in various tumor cells in vitro and in vivo. Intrinsic protumorigenic and antitumorigenic properties thus seem to be highly dependent on the tumor type and its stromal composition, including cellular components and the ECM. Besides the tumor type, the origin of the MSCs and their in vitro cultivation during certain experimental set-ups may also influence the differential role of naive MSCs in a particular tumor (reviewed in Ciavarella et al. 2011; Grisendi et al. 2011).

Although doubts still exist whether naive MSCs themselves indeed have a suppressive effect on tumor growth when used as an isolated therapy, evidence is accumulating that MSCs could be used as an ideal carrier for anticancer genes/drugs (Loebinger et al. 2009; Loebinger and Janes 2010). Gene delivery poses a great challenge in highly invasive and infiltrative tumors, such as malignant gliomas or pulmonary cancer metastases, where tumor cells infiltrate into surrounding tissue in strands or as single cells. Genetically engineered MSCs are capable of producing and locally releasing specific anticancer agents (Sasportas et al. 2009). Such transgene strategies potentiating the MSC antitumor effect were recently reviewed by Shah (2012), who presented examples of MSC clones that have been genetically modified to express target exogenous genes and/or secrete desired molecules as drugs for constant treatment of different tumors locally (Binello and Germano 2012). These molecules include interleukins, interferons, chemical prodrugs, antiangiogenic agents and oncolytic viruses. All these have so far been used in various animal studies, but to our knowledge, no transgenic MSCs have been used in humans. Recently, Grisendi et al. (2011) proposed that transgenic killer MSCs, so-called mesenkillers, produced by retroviral (adenoviral or lentiviral) transduction of MSCs could besides tumor cells also target tumor stromal cells, for example tumor-associated fibroblasts, themselves originating from MSCs (Dominici et al. 2009). Direct injections of transgenic MSCs at the tumor sites gave satisfactory results in mouse and rat models of different carcinomas, melanoma and glioma. Yet, for invasive types of cancer a novel therapeutic approach using a combination of MSCs as intermediate carriers of conditionally replicating

adenoviruses (CRAds) emerged (Stoff-Khalili et al. 2007). Because MSCs support amplification of CRAds, more efficient tumor cell elimination was observed in distant tumors compared with direct viral injection. This two-component system needs further optimization and fine-tuning for higher efficiency. Together with other examples summarized by Grisendi et al. (2011) and Shah (2012), genetically modified MSCs with active homing potential could be potentially used for treating infiltrative tumors that cannot be cured by conventional drugs.

Not only BM-MSCs but also AT-MSCs are gaining increasing interest and can be applied for cancer treatment, since they have the same potential and are easily available for autologous and allogenic transplantations, as discussed above. First reported by Kucerova et al. (2007) as enhancing tumor cell proliferation, AT-MSCs were later shown to induce pancreatic carcinoma cell necrosis in vitro and display a long-lasting inhibitory effect on tumor growth (Cousin et al. 2009).

15.3.4 Potential Treatment of Glioma

The first step in treating glioma in most cases involves neurosurgical removal of the main tumor mass, followed my chemotherapy and radiotherapy, all of which do not significantly prolong patient survival. The main challenge in treating glioma remains targeting single tumor cells that have deeply infiltrated into the normal surrounding brain parenchyma. Owing to the observation that normal stem cells, including MSCs, have the ability to infiltrate the blood–brain barrier and via tropism track to the individual tumor cell intracranially (Aboody et al. 2000; Sasportas et al. 2009), central nervous system tumors have been extensively investigated as the candidate tumor type for development of such (stem) cell-based therapeutics. So far, three different stem cell types, embryonic stem cells, NSCs and MSCs have been studied and used as vehicles for delivering various therapeutic agents to glioma. Nakamizo et al. (2005) and Bexell et al. (2009) clearly showed that when MSCs are injected into the tumor they have the ability to migrate towards distant/outmigrated glioma cells. Thus, they could be exploited to deliver toxic substances to brain tumor cells that have already spread into the normal brain parenchyma while sparing normal brain cells (Bexell et al. 2010). Recent examples of the application of MSCs in a preclinical set-up for glioma treatment (reviewed in Grisendi et al. 2011; Binello and Germano 2012; Shah 2012) are presented in Table 15.1 and are discussed in the following sections.

15.3.4.1 Cytokines

In an in vitro study by Nakamura et al. (2004), the soluble factors released from glioma cells clearly stimulated the migration of rat MSCs. Similarly, MSCs overexpressing IL-18 and IL-12 have been used as therapeutic vectors to treat glioma and other tumors (Stagg et al. 2004). Hamada et al. (2005) showed that intratumoral inoculation of adenovirally transduced MSCs producing IL-2 significantly

Table 15.1 Applications of mesenchymal stem cells (*MSCs*) as vehicles for delivery of therapeutic agents to glioma

Authors	Model	Modality	Key findings
Cytokines			
Nakamura et al. (2004) Hamada et al. (2005)	Rat 9L glioma cell line Rat 9L glioma in rats	Rat MSCs Rat MSC-IL-2	In vitro: Inhibition of proliferation of 9L cells by co-cultivation with MSCs due to soluble factors produced by MSCs. MSC *tropism* to 9L cells In vivo: MSCs possess great migratory ability and *tropism* to glioma; MSCs were found on the border between tumor and normal parenchyma and also infiltrated into the tumor. Intratumoral injection of MSC-IL-2 significantly prolonged survival by paracrine and autocrine loops
Nakamizo et al. (2005)	Human U87MG glioma cell line Human U87MG, U251 and LN229 glioma in nude mice	hMSCs hMSC-IFN-β	In vitro: hMSCs possess great migratory ability and *tropism* to glioma due to PDGF, EGF and SDF-1α. *Growth inhibition* of U87MG glioma cells by IFN-β released by transfected hMSCs In vivo: hMSCs migrate towards gliomas after intracranial injection. *Prolonged survival* by hMSC-IFN-β delivered regionally
Oncolytic viruses			
Miletic et al. (2007)	Rat 9L glioma in Fischer rats	Rat BM-TIC-HSV-tk and ganciclovir	In vitro: Gap junction formation between BM-TICs and 9L cells. Gap junctions make possible a *bystander effect*. After addition of ganciclovir to co-cultures of glioma cells and BM-TIC-HSV-tk, transduced BM-TICs exerted a bystander effect on glioma cells. In vivo: BM-TICs infiltrated solid parts as well as the border of glioma. In situ injection of BM-TIC-HSV-tk confirmed a *bystander effect* on glioma and shoved significantly *prolonged survival*
Uchibori et al. (2009)	Rat L9 glioma in nude mice	Rat MSC-HSV-tk and ganciclovir Rat MSC-VP	In vivo: MSC-VP accumulated at the site of tumors after intravascular injection, followed by in situ gene transfer to tumors without transduction of normal organs. *Bystander-mediated glioma cell killing:* distant intravascular injection of a combination of MSC-VP and MSC-HSV-tk exerted a stronger effect than MSC-HSV-tk alone

(continued)

Table 15.1 (continued)

Authors	Model	Modality	Key findings
Sonabend et al. (2008)	Human U87MG glioma cell line Human U87MG glioma in nude mice	hMSC-CRAd	In vitro: hMSC-CRAd effectively migrated in vitro and released CDAds that infected U87MG cells In vivo: *Bystander-mediated glioma cell killing*. hMSCs migrate to the tumor and deliver CRAds even to distant glioma cells efficiently and are even moe efficient than intracranial injection of CRAd-CXCR4-5/3 plasmid alone
Yong et al. (2009)	Human U87MG and U251-V121 glioma in athymic mice	hMSC-Δ24-RGD	In vivo: Prolonged survival and tumor eradication. Inhibited tumor growth and increased short-and long-term survival of mice, significant eradication of the tumor in a subset of treated mice compared with controls
Recombinant antibodies			
Balyasnikova et al. (2010)	Human U87MG-EGFRvIII glioma cell line Human U87MG-EGFRvIII glioma in athymic mice	hMSC-scFvEGFRvIII hMSC-scFvEGFRvIII fused with PDGFR	In vitro: *Delayed growth* of U87MG-EGFRvIII cells in co-culture with MSC-scFvFRvIII. Fusion with PDGFR domain increased expression of scFvEGFRvIII. hMSCs displayed *enhanced binding* to U87-EGFRvIII cells. In vivo: *Prolonged survival* and *tumor shrinkage*. Enhanced retention and inhibited tumor growth. Intracranial co-injection of U87MG-EGFRvIII and hMSC-scFvEGFRvIII significantly improved survival. Fusion with PDGFR *improved targeting* of glioma cells
Proapoptotic drugs			
Kim et al. (2008)	Human U87MG glioma in nude mice	UCB-hMSC-sTRAIL	In vivo: *Inhibition of tumor growth* and *prolonged survival* of glioma-bearing mice. MSC-based sTRAIL gene delivery had more therapeutic efficacy than direct injection of adenovirus bearing sTRAIL

(continued)

Table 15.1 (continued)

Authors	Model	Modality	Key findings
Menon et al. (2009)	Human U87MG glioma cell line Human U87MG glioma in nude mice	BM-hMSC-sTRAIL	In vitro: Human glioma cells *stimulated migration* of BM-hMSC-sTRAIL and BM-hMSC-sTRAIL showed the same *tropism* as untransduced BM-hMSCs. BM-hMSC-sTRAIL promoted U87MG *cell death* In vivo: Effective *inhibition of intracranial U87MG glioma* tumor growth. Significantly *longer mouse survival*.
Kauer et al. (2012)	Human U87MG glioma in nude mice	sECM-NSC-sTRAIL sECM-hMSC-sTRAIL	In vitro: *Eradication of residual tumor cells* by inducing caspase-mediated apoptosis In vivo: *Delayed tumor* regrowth and significantly *longer mouse survival*. Encapsulation of stem cells increased their retention time in the GBM resection cavity
Shah (2012)	Human Gli36 U87MG, U251, Gli79, A172, LN229 and $CD133^+$ GBM8 glioma in nude mice	mNSC-sTRAIL PI3K/mTOR inhibitor PI-103	In vitro: In co-cultures the inhibitor PI-103 augmented the response of glioma cells to mNSC-sTRAIL. PI-103 caused G0–G1 arrest in the cell cycle—*inhibition of proliferation and invasion* In vivo: Significant *attenuation of orthotopic tumor growth and tumor volume* by aystemic PI-103 delivery combined with mNSC-sTRAIL
Hingtgen et al. (2012)	Human U87MG-fLuc, U251 and 293T glioma in nude mice	mNSC-SM7L mNSC-sTRAIL	In vitro: Diagnostic domain of SM7L produced robust photon emission. Therapeutic domain affected GBM cells and modulated p38 MAPK and ERK pathways In vivo: Real-time monitoring confirmed mNSC-SM7L improved pharmacokinetics and *attenuated progression of peripheral and intracranial human GBM xenografts*. mNSC-SM7L antitumor efficacy was augmented by caspase activation, and apoptosis was induced by mNSC-sTRAIL delivery

(continued)

Table 15.1 (continued)

Authors	Model	Modality	Key findings
Bagci-Onder et al. (2011)	Human Gli36, U87MG, U251, Gli79, A172, LN229 and $CD133^+$ GBM8 glioma in nude mice	mNSC-sTRAIL mMSC-sTRAIL PI3K/mTOR inhibitor PI-103	In vivo: Systemic delivery of PI-103 combined with mMSC/mNSC-sTRAIL significantly *reduced tumor volume* as compared with PI-103 treatment alone
Antiangiogenesis			
Bexell et al. (2009)	Rat N29 and N32 glioma transplacental injection into pregnant Fischer344 rats	Rat BM-MSCs	In vivo: *Migratory ability*—BM-MSCs infiltrated the invasive glioma extensions and reduced distant tumor microsatellites highly specific for tumor tissue. *Neovascularization*—grafted BM-MSCs integrated into tumor vessel walls showed an expression profile and perivascular location typical for pericytes *No effects on survival of rats*—BM-MSCs had no influence on tumor microvessel density; the antiangiogenic drug sunitinib reduced the numbers of BM-MSCs migrating within tumors

MSC-IL-2 MSCs modified to produce the human IL-2 gene by adenovirus-mediated gene transduction, *hMSC* human MSC, *PDGF* platelet-derived growth factor, *EGF* epidermal growth factor, *SDF-1α* stromal-cell-derived factor 1α, *IFN-β* interferon-β, *hMSC-IFN-β* human MSCs modified to produce the human interferon-β gene by adenovirus-mediated gene transduction, *BM-TIC* bone-marrow-derived tumor-infiltrating cells, *HSV-tk* thymidine kinase of herpes simplex virus, *BM-TIC-HSV-tk* bone-marrow-derived tumor-infiltrating cells transduced with retroviral vector expressing the thymidine kinase suicide gene of herpes simplex virus, *MSC-VP* MSCs nucleofected with proviral plasmids, *CRAd* replication-competent oncolytic adenovirus, *hMSC-CRAd* human MSCs infected with replication-competent oncolytic adenovirus, *Δ24-RGD* oncolytic adenovirus, *hMSC--Δ24-RGD* human MSCs infected with Δ24-RGD, *EGFRvIII* epidermal growth factor receptor variant III, *scFv* single-chain antibody, *U87MG-EGFRvIII* U87MG cells expressing epidermal growth factor receptor variant III, *hMSC-scFvEGFRvIII* human MSCs expressing scFv against epidermal growth factor receptor variant III, *PDGFR* platelet-derived growth factor receptor, *fLuc* firefly luciferase, *UCB-hMSC* umbilical-cord-blood-derived human MSCs, *sTRAIL* secretable form of TNF-related apoptosis-inducing ligand, *UCB-MSC-sTRAIL* adenovirally transduced umbilical-cord-blood-derived human MSCs with the secretable form of TNF-related apoptosis-inducing ligand, *BM-hMSC* bone-marrow-derived human MSCs, *BM-hMSC-sTRAIL* lentivirally transduced bone-marrow-derived human MSCs with the secretable form of TNF-related apoptosis-inducing ligand, *sECM* synthetic extracellular matrix, *mNSC* mouse neural stem cell, *sECM- mNSC-sTRAIL* synthetic extracellular matrix encapsulated mouse neural stem cells bearing the secretable form of TNF-related apoptosis-inducing ligand, *sECM-hMSC-sTRAIL* synthetic extracellular matrix encapsulated human MSCs bearing the secretable form of TNF-related apoptosis-inducing ligand, *mNSC-sTRAIL* lentivirally transduced mouse neural stem cells with the secretable form of TNF-related apoptosis-inducing ligand, *PI3K* phosphoinositide 3-kinase, *mTOR* mammalian target of rapamycin, *SM7L* multifunctional targeted anticancer molecule, *mNSC-SM7L* lentivirally transduced mouse neural stem cells with SM7L, *mMSC-sTRAIL* lentivirally transduced mouse MSCs with the secretable form of TNF-related apoptosis-inducing ligand

prolonged survival of glioma-bearing rats. This suggested that rat survival may depend on a direct antitumor effect of IL-2, which may act even in an autocrine manner on MSCs localized around the tumor edge to express angiopoietin-1. Likewise, Nakamizo et al. (2005) engineered human MSCs expressing interferon-β and injected them into nude mice with human U87MG and LN229 intracranial gliomas. Mice treated with human MSCs expressing interferon-β had a significantly higher survival rate than non-treated mice.

15.3.4.2 Prodrug Activation Mediated by Mesenchymal Stem Cells

Another use of MSCs as 'pharmacologic pumps' refers to MSCs equipped with nontoxic prodrugs which on activation display a bystander tumor-cell-killing effect. An example of a such drug is cytosine deaminase, which converts the nontoxic 'prodrug' 5-fluorocytosine into the readily diffusible toxic 5-fluorouracil. Engineered human MSCs were shown to support replication of adenovirus bearing herpes simplex virus (HSV) thymidine kinase (TK) or expressing the TK gene themselves. Following the systemic administration of ganciclovir or injection of it into the tumor or its vicinity, a bystander-effect-mediated glioma cell killing was demonstrated by Miletic et al. (2007) and Kucerova et al. (2011). Necrosis in infiltrated solid parts as well as at the borders of rats' glioma was observed, proving the significant tumor volume reduction and high therapeutic efficacy of TK. Later Uchibori et al. (2009) developed MSCs that produced progeny retroviral HSV-TK vectors locally. Although on HSV-TK–MSC co-injection into mice subcutaneously together with 9L glioma cells, followed by a systemic application of ganciclovir, the tumor growth was significantly suppressed, the same therapeutic effect was unfortunately not observed when modified MSCs were injected into pre-established 9L tumors.

15.3.4.3 Oncolytic Viruses

MSCs have also been used as vehicles for local release of intact conditionally replicating oncolytic adenoviruses (CRAds). Sonabend et al. (2008) reported that MSCs producing CRAd expressing CXCR4 migrate to gliomas and mediate a bystander killing effect on infected glioma cells in an enhanced manner when injected distant to the tumor. In a similar study, Yong et al. (2009) demonstrated that MSCs producing oncolytic adenovirus Δ24-RGD enhance systemic migration towards glioma cells and show increased ability to inhibit glioma growth and prolong survival of mice.

15.3.4.4 Recombinant Antibodies

Half of the GBM samples show expression of the truncated (mutated) form of EFGRs, i.e. EGFRvIII on the cell surface, making it an ideal target for antibody-mediated drug delivery. Balyasnikova et al. (2010) therefore genetically

modified MSCs to express a single-chain antibody (scFv) to EGFRvIII on their surface. MSCs expressing scFv against EGFRvIII showed preference for binding EGFRvIII-expressing glioma cells in vitro and improved retention time of EGFRvIII-expressing glioma cells in vivo. Significantly increased survival was noted in animals into which MSCs expressing scFv had been transplanted and was even increased on administration of a second dose of MSCs expressing scFv.

15.3.4.5 Proapoptotic Drugs

Secchiero and Zauli (2008) reported on recombinant-TRAIL-induced human MSC migration via ERK1/2 which does not affect proliferation, lineage differentiation and most importantly does not induce an apoptotic response in MSCs. BM-MSCs, AT-MSCs and umbilical-cord-derived MSCs have been modified with several members of the death ligand family, including TNF-α and TRAIL. The latter is a powerful anticancer molecule, which specifically kills tumor cells expressing death receptors (DR), but not the bearing MSC vehicle. The secretable form of TRAIL (sTRAIL) consists of a fused extracellular TRAIL domain and the human Flt3 ligand's extracellular domain. The latter binds to the Flt3 RTK and is efficiently secreted into the producer cell's immediate microenvironment, exhibiting higher cytotoxicity with regard to glioma cells than the native TRAIL protein (Shah et al. 2005). Yet both of them induce caspase-mediated apoptosis in established GBM cell lines as well as in GBM stem cells in vitro. With use of a highly malignant and invasive human GBM stem cell model and real-time imaging on neuropathology samples, sTRAIL-bearing MSCs migrating extensively to brain tumors were confirmed to have a profound antitumor effect in vivo. This way, therapeutic efficacy of sTRAIL and the potential of MSCs as delivery vehicles targeting GBM stem cells in a clinical set-up was ultimately demonstrated (Sasportas et al. 2009).

Kim et al. (2008) reported on therapeutic effect of TRAIL-bearing BM-MSCs and umbilical-cord-derived MSCs when they are transplanted into U87MG glioma-bearing animals. This way, prolonged animal survival was additionally confirmed by Menon et al. (2009). In vitro, MSC–TRAIL targeting of tumor cells performed well with the proteasome inhibitor bortezomib (Grisendi et al. 2010), where killing activity was associated with the activation of caspase-8. This has also been applied in various experiments of U87MG GBM xenografts in nude mice by Shah (2012), which confirmed similar effects of both normal NSCs and MSCs on GBM progression.

Furthermore, Bagci-Onder et al. (2011) evaluated the therapeutic efficacy of combining NSCs producing sTRAIL with a novel PI3K/mammalian target of rapamycin inhibitor (PI-103) in glioma cell lines and primary CD133 glioma-initiating cells. Enhanced response of glioma cells in vitro and in an orthotopic glioma mouse model was observed compared with PI-103 treatment alone. During glioma cell elimination via engineered NSCs, the latter preserved the ability to regenerate the damaged tissue site, implying MSCs may have a similar potential. Indeed, the animal model experiments confirmed that injection of MSCs expressing neurofilament or neuron-specific enolase into a rat model of Parkinson's disease did result

in a 50 % remission of normal behaviour (Sadan et al. 2009). Genetically modified MSCs, which have the potential to renew brain function, may therefore prove efficient during neuron regeneration after brain tumor elimination.

Also various stem cell lines were engineered bearing TRAIL and luciferase (SRLOL2TR) to ease diagnostic imaging and show differences in the levels and duration of TRAIL secretion between stem cell lines, which translates into their significant variability in tumor cell killing (Hingtgen et al. 2010). Recently, new models incorporating SM7L biology that simultaneously enhance tumor cell killing and permit diagnostic tracking were created (Hingtgen et al. 2012). SM7L comprises the antitumor cytokine MDA-7/IL-24 therapeutic secretory domain and diagnostic domain for non-invasive tracking. Its therapeutic domain showed marked antitumor efficacy and significant modulation of the p38 MAPK and ERK pathways, confirming improved stem-cell-delivered SM7L-mediated pharmacokinetics that results in attenuated progression of established peripheral and intracranial human GBM xenografts. Furthermore, the antitumor efficacy for stem-cell delivered SM7L was increased in vitro *and* in vivo by concurrent activation of caspase-mediated apoptosis induced by adjuvant MSC–sTRAIL delivery.

Yet another approach employing the sTRAIL strategy to target GBM has been recently reported by Kauer et al. (2012). This approach uses therapeutic sTRAIL-bearing stem cells encapsulated in biodegradable, synthetic ECM to target human GBM in mouse. With use of multimodal imaging, the first quantitative surgical debulking of human GBM tumors was observed, as synthetic ECM encapsulation of engineered MSCs permitted release of tumor-selective sTRAIL this way delaying tumor regrowth and significantly increasing survival of mice. This study demonstrates that the efficacy of encapsulated therapeutic stem cells may have implications for developing effective GBM therapies.

15.3.4.6 Angiogenesis and Vascular Normalization

In experimental glioma, BM-MSCs can act as pericyte-like migratory vehicles and localize to tumor vasculature on intratumoral implantation as suggested by Bexell et al. (2009, 2010). This could be exploited to targeted vascularized tumors with MSCs expressing antiangiogenic agents to normalize vascularization and enhance the antitumor immune response. For example, Bexell et al. have shown that a single administration of stem cell antiangiogenic thrombospondin-1 markedly reduces tumor vessel density and significantly inhibits progression of highly malignant human glioma xenografts in mice.

In all the reports mentioned, the molecular mechanisms of exogenous MSC–GBM interaction underpinning the observed phenotypic characteristics are not known. Therefore, we employed the cell-based model, mimicking tumor therapy by using naive MSCs to study the interactions with various different GBM cell lines (Motaln et al. 2012) (Fig. 15.3). We demonstrated that on growing MSCs and U87MG GBM cells in co-cultures, the latter induced MSC proliferation and invasion, whereas the opposite effect was observed in U87MG cells, which in

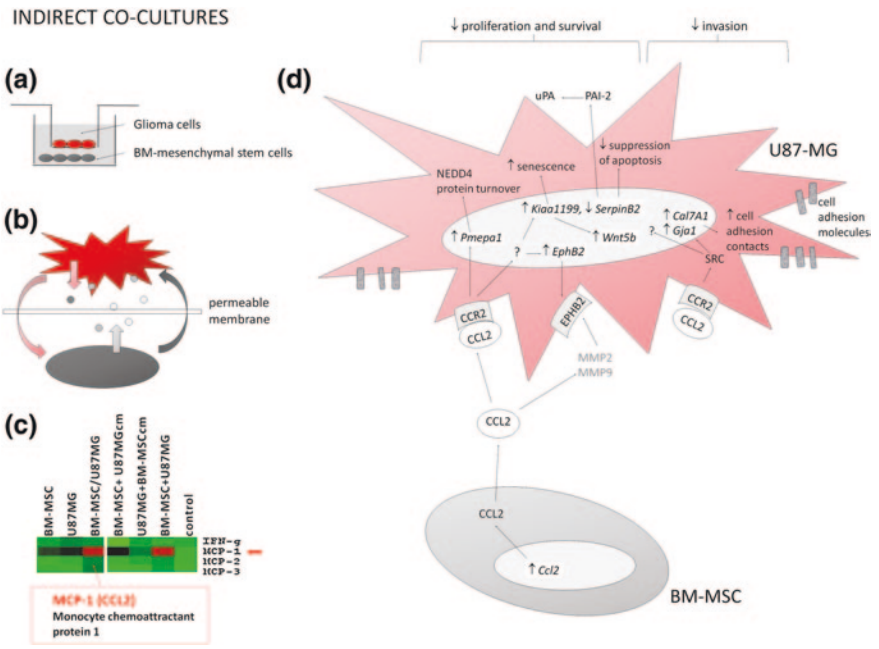

Fig. 15.3 MSCs and glioblastoma cells in indirect co-cultures in vitro: cytokine and gene expression profiling. Cytokine profiling of an indirectly co-cultured U87MG glioblastoma cell line and stromal BM-MSCs. **a** Cells were cultured in indirect co-cultures using Transwells (Corning Life Sciences). **b** In indirect co-cultures cells do not have direct cell contact and paracrine signalling between different cell types is dependent on the feedback loops. Indirect co-culture may mimic cell delivery during tumor cell therapy. **c** Cytokines were profiled using a cytokine antibody array (RayBiotech), a 79-cytokine panel, where the level of CCL2/monocyte chemoattractant protein 1 (*MCP-1*) was identified as most significantly increased in direct (*BM-MSC+U87MG*) and indirect (*BM-MSC/U87MG*) co-cultures of MSCs and U87MG cells. **d** Transcriptomic analysis was performed on both cell types in co-cultures in relation to monocultures, and revealed the potential effects on the cytokines, including the most relevant CCL2/MCP-1. A generalized scheme of BM-MSC and U87MG cell cross-communication, triggered by CCL2/MCP-1 is shown as analysed by the Biomine search engine (http://biomine.cs.helsinki.fi/search/) using the three most relevant upregulated genes as the query. These data explain the observed phenomenon that MSCs decrease the proliferation and survival and increase the senescence and impair the invasion of GBM cells. The transcriptomic analysis showed that invasion impairment seems to be regulated through the increased presence of cell adhesion contacts, but not by a migratome and proteases. *U87MGcm* conditioned medium from U87MG, *BM-MSCcm* conditioned medium from BM-MSCs. (**d** Adapted from Fig. 6 in Motaln et al. 2012)

co-culture even underwent senescence. By cytokine profiling and whole genome messenger RNA analysis of MSC and U87MG cells, we identified CCL2/monocyte chemoattractant protein 1 collectively as most significantly regulated chemokine during this paracrine signalling and confirmed its role in U87MG invasion. Additionally, the data mining analysis revealed several other genes and signalling pathways that could be affected by CCL2/monocyte chemoattractant

protein 1 acting in both cell lines in the opposite manner. For example, increased levels of cell–cell adhesion molecules seem to be responsible for lower GBM invasion, whereas in MSCs, increased motility and invasion could be due to increased levels of various cytokine (IL-8, CXCL1 and CXCL6) related signalling pathways.

15.4 Conclusion

Despite showing success in the initial studies of experimental therapies, ssome potential obstacles in translating stem cell therapies into the clinic still exist. We need to account for the intrinsic contribution of MSCs to the protumorigenic stroma as well as their immunosuppressive functions, even if they are only vectors for gene delivery. It is clear that new modalities of cancer treatment are urgently needed and that stem cell research should soon be pushed to the clinic. Optimistically, four clinical trials using naive normal stem cells for tumor therapy are in progress (Shah 2012). Yet in brain tumors, the stem cell type and the best route of administration to treat this malignancy need to be optimized. Furthermore, we need to determine which therapeutic target should be used to result in the best possible therapeutic effect in brain tumor patients. Nevertheless, we strongly believe that only astute investigation of naive and engineered MSCs may lead to the development of efficient therapies for cancer patients.

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Chapter 16
Implications of Glioblastoma Stem Cells in Chemoresistance

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Abstract Cancer stem cells, identified in gliomas as well as in other cancer types, are defined as undifferentiated cells with the ability to self-renew, differentiate to multiple lineages, and initiate tumors that mimic the parent tumor. Glioma stem cells are resistant to radiotherapy and chemotherapy and play a major role in repopulating tumors following treatment. An integrated view of the markers, niche, and vasculature, as well as therapeutic resistance, is essential to understand the key processes occurring within glioma stem cells and, therefore, to identify potential therapeutic targets that can lead to the development of more effective cancer treatments.

Keywords Glioblastoma • Cancer stem cells • Chemoresistance • Tumorigenesis

16.1 Introduction

For a long time, tumorigenesis has been, known to resemble organogenesis, and most tumors are heterogeneous, containing several phenotypically and functionally different cell types (Faria et al. 2006). Tumor cell heterogeneity may be a consequence of clonal evolution caused by genetic instability and/or of maturation/differentiation of stem-like cells, frequently called cancer stem cells (CSCs)

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or tumor-initiating cells (Shackleton et al. 2009). Current data demonstrate that clonal evolution and CSC-directed development may not be reciprocally exclusive and both mechanisms may assist in the generation of tumor cell heterogeneity (Anderson et al. 2011; Notta et al. 2011). Although there are still discussions about CSCs as a concept, there is no dispute about malignant tumors being immortal at the population level. Thus, it will be appealing and essential to identify, as well as to describe, the immortal subpopulations of cancer cells.

The theme of CSCs is controversial not because CSCs do not exist, but because misunderstandings and inadequate assays have generated numerous contradictions. The designation of CSCs must be intended as a functional characterization. Indeed, as normal stem cells are defined by their stem cell activity, CSCs must be defined in functional assays by their capacity to produce serially transplantable tumors that recapitulate the cellular and histostructural heterogeneity of the parent tumor. Simple marker expression and in vitro analysis are not enough to label any cancer cells as CSCs. In addition, several CSCs described so far have not been subjected to serial transplantation assays; instead, only tumor frequency and/or tumor mass were used to evaluate the CSC activity. Such methods might as well analyze tumor progenitors rather than CSC activity owing to the high proliferative competence of the progenitor cell population. Serial tumor transplantation assays, particularly at low cell amounts, should allow discrimination between long-term CSCs and fast proliferating tumor progenitors, both of which have tumor-initiating competence.

Accordingly, the brain tumor stem cell theory holds that glioma stem cells (GSCs) generate all the cells of the tumor and therefore represent the fundamental, precise targets for successful treatment in order to avoid recurrence (Nakano and Kornblum 2006). The concept that glioma tumors originate from transformed neural stem cells (NSCs) was initially encouraged by the finding that brain tumors express nestin, an intermediate filament encountered in NSCs (Dahlstrand et al. 1992; Tohyama et al. 1992). In this GSC model, brain tumor stem cells arise from oncogenic mutations in NSCs. This hypothesis was sustained by some observations, as follows: gliomas can occur near the lateral ventricles, a site bordering NSCs that occupy the subventricular proliferative zone; NSCs proliferate enough to make them vulnerable to transformation; and NSCs and GSCs have similar mechanisms for proliferation and survival (Sanai et al. 2005; Hadjipanayis and Van Meir 2009).

Substantiation of the GSC model first came from numerous laboratories (Ignatova et al. 2002; Hemmati et al. 2003; Singh et al. 2003). The assays established biological resemblances among brain-tumor-initiating cells and NSCs through the adoption of the neurosphere culture model (floating spheres of cells). NSCs were initially isolated and enriched from the adult brain through the use of neurosphere cultures (Reynolds et al. 1992). NSCs differ from other cells in the brain by their capacity to be cultured as neurospheres in serum-free media with the addition of epidermal growth factor (EGF) (Reynolds and Weiss, 1992) or basic fibroblast growth factor (Chiang et al. 1996), or both (Chow et al. 2000). Neurospheres are able to differentiate into neurons, astrocytes, and oligodendrocytes after removal of growth factors. Similarly, cells originating from brain tumors form serially passaged clonal neurosphere cultures in serum-free media and, after

withdrawal of growth factors, differentiate into multiple lineages to recapitulate the parental tumor. Therefore, GSCs behave, in vitro, in a manner similar to neural progenitor cells since they react to the same mitogens and express similar markers.

The GSC theory was further substantiated when it was demonstrated that glioblastoma (GBM)-derived neurosphere cultures were tumorigenic on xenotransplantation into immunodeficient mice (Galli et al. 2004). Moreover, it was shown that tumor cells expressing CD133 (an established marker of human NSCs), when sorted from patient samples, generated tumors in immunodeficient mice, whereas the $CD133^{-}$ cells did not (Singh et al. 2004a). The GSC theory is accepted and recognized on the basis of the following features: GSCs may derive from transformed NSCs or progenitor cells and possess the ability to self-renew, generate tumors upon xenotransplantation, and give rise to a heterogeneous population of cells equivalent to their parent tumors. More recent analysis revealed that the capacity of glioma tumors to generate neurospheres in culture is a self-sufficient predictor of clinical outcome (Pallini et al. 2008; Laks et al. 2009). These data offer further confirmation that GSCs play an essential role in tumor progression and aggressiveness. However, the GSC theory is rather a hypothesis, and both the definition and terminology continue to be discussed. Therefore, some authors adopt the less compromising terms "brain-tumor-initiating cells" and "brain tumor stem-like cells".

16.1.1 GSC Markers

CSC studies would not have advanced without the pioneering work executed in the hematopoietic field via fluorescence-activated cell sorting to trace the lineage of cells, using a variety of cell surface markers, which provide the capacity to identify and isolate a stem cell population with long-term self-renewing capacity (Spangrude et al. 1988).

Knowledge of GSCs has been enriched on the basis of functional tests, including side population (SP) analysis (Bleau et al. 2009a; Broadley et al. 2011; Golebiewska et al. 2011) and neurosphere assays (Bonavia et al. 2011; Pastrana et al. 2011), or cell surface molecules, such as CD133 (Fig. 16.1), also known in humans and rodents as prominin-1 (Singh et al. 2004b; Eyler et al. 2011), CD15, also called Lewis X and stage-specific embryonic antigen 1 (SSEA-1) (Fig. 16.1) (Son et al. 2009), EGF receptor (EGFR; Inda et al. 2010; Mazzoleni et al. 2010); integrin α_6 (Lathia et al. 2010), A2B5 (Nunes et al. 2003), and CD44 (Anido et al. 2010). Central to the GSC hypothesis is the capacity to use these surface markers to isolate the putative stem population within the heterogeneous tumor mass and functionally corroborate the stem cell phenotype of this population versus the non-stem-cell population (Shackleton et al. 2009). The most commonly used GSC markers are SSEA-1, A2B5, and CD133.

SSEA-1 (Fig. 16.1) is a carbohydrate antigen connected with glycolipids and glycoproteins that is expressed by NSCs derived from human embryonic stem cells, embryonic NSCs, and GSCs (Barraud et al. 2007; Pruszak et al. 2007; Son

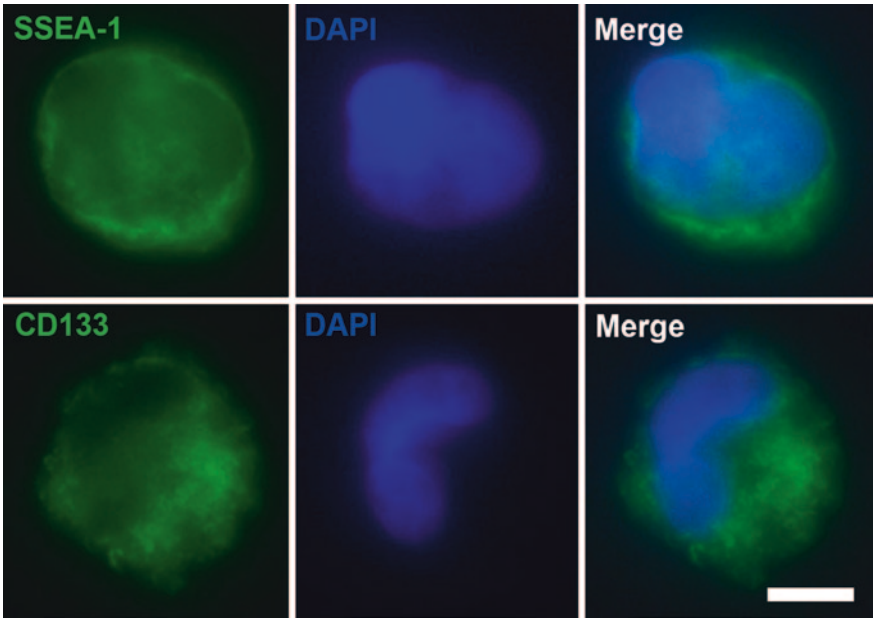

Fig. 16.1 Glioblastoma stem cells express CD133 and stage-specific embryonic antigen 1 (*SSEA-1*) in the plasma membrane. Immunocytochemistry analysis of CD133 and SSEA-1 expression in a cancer stem cell line derived from primary cultures of glioblastoma. The images show single cells. *DAPI* 4′,6-diamidino-2-phenylindole, *scale bar* 5 μm

et al. 2009). SSEA-1$^+$ GBM populations express higher levels of stem cell genes, such as *SOX2* and *BMI1*, and are able to self-renew and differentiate into neural lineages (Son et al. 2009). SSEA-1$^+$ cells isolated from GBMs revealed higher levels of tumor initiation and development in mouse intracranial xenograft models in comparison with SSEA-1$^-$ cells. Moreover, 95 % of primary GBMs analyzed presented a subpopulation of SSEA-1$^+$ cells (Son et al. 2009).

A2B5 is a cell surface ganglioside initially described in neural precursor cells in the adult human brain (Nunes et al. 2003), although it was demonstrated that a portion of NSCs isolated from the human embryo subventricular zone is A2B5$^+$. A2B5 was also identified in NSCs derived from human embryonic stem cells (Pruszak et al. 2007). Finally, it was demonstrated that GSCs express A2B5 (Ogden et al. 2008; Tchoghandjian et al. 2010), as A2B5$^+$ cells are able to initiate intracranial tumor formation, whereas A2B5$^-$ cells are not (Tchoghandjian et al. 2010). Both A2B5$^+$/CD133$^+$ and A2B5$^+$/CD133$^-$ GSC fractions are able to give rise to neurospheres in culture and to generate a tumor mass in intracranial models. These data indicate that A2B5 might indeed be an additional GSC marker.

CD133 (Fig. 16.1), a cell membrane glycoprotein, was first identified on a hepatoma cell surface and is generally expressed by hematopoietic stem cells (Yin et al. 1997), endothelial precursor cells (Salven et al. 2003), and normal stem cells

(Uchida et al. 2000). It was shown that $CD133^+/CD34^+$ cells are functionally nonadherent precursors that have the ability to differentiate into mature endothelial cells and might be involved in postnatal lymphangiogenesis and/or angiogenesis (Salven et al. 2003).

Even though CD133 has been adopted to identify and isolate human stem cells from different tissues owing to its rapid downregulation on cell differentiation, its role remains unclear (Corbeil et al. 2000; Rafii 2000). CD133 localization in membrane protrusions implies a participation in the dynamic organization of such protrusions and, therefore, in the mechanisms controlling cell polarity, migration, and interaction of stem cells with neighboring cells and/or the extracellular matrix (ECM). Experimental evidence, however, is still lacking to prove this. Moreover, it is also unclear whether CD133 has participates in self-renewal and differentiation of stem cells.

It was demonstrated that the $CD133^+$ population of GBM has stem cell properties in vitro (Shmelkov et al. 2004) and leads to tumor formation and growth in vivo (Galli et al. 2004; Shmelkov et al. 2004), robustly indicating that $CD133^+$ cells may be the brain-tumor-initiating cells. The incidence of this expression marker is, at most, 30 % in GBM (Dirks 2008). High CD133 expression was found to be associated with poor prognosis and low survival rates of GBM patients (Pallini et al. 2008; Rebetz et al. 2008; Zeppernick et al. 2008). Establishment of both GSCs in vitro and the occurrence of $CD133^+/Ki67^+$ cells might have a prognostic significance. Patients whose tumor biopsies formed GSCs in vitro expressing CD133 and Ki67 had a poor outcome and a shorter survival in comparison with patients with tumors that did not generate GSCs (Pallini et al. 2008). Together, this means that the capacity to form in vitro GSCs might discriminate a poor prognostic subtype of GBM that leads to disease progression and recurrence.

There are considerable doubts surrounding the use of CD133 as a marker for GSCs. Even though CD133 has been extensively adopted to enrich GSCs (Singh et al. 2004b; Eyler et al. 2011), tumorigenic cells are found in both $CD133^+$ and $CD133^-$ cell fractions in some gliomas (Beier et al. 2007), and certain $CD133^+$ glioma cells may not exhibit elevated tumor-initiating ability. Indeed, the $CD133^-$ cell population from some GBMs may actually possess long-term self-renewing tumor-initiating cells (Chen et al. 2010). Although it has been admitted that there is a lineage relationship between $CD133^+$ and $CD133^-$ glioma cells, the two subpopulations may have different cells of origin (Lottaz et al. 2010). Additional studies are necessary to resolve these contradictory data. It was suggested that GBMs exhibit several individual populations of linearly correlated $CD133^-$ cells representing different stages of differentiation, with some $CD133^-$ cells producing aggressive tumors evidencing both $CD133^+$ and $CD133^-$ cells and some others evidencing only $CD133^-$ cells, perhaps by symmetric stem cell renewal, producing slow-growing circumscribed tumors of $CD133^-$ cells (Chen et al. 2010). These findings highlight the complexity of the CSC hypothesis as well as the field's appreciation that no single marker is sufficient to identify all GSC populations.

Some reports evidenced that microRNA (miR)-137 and miR-124 are downregulated in GSCs relative to nonneoplasic brain tissue, and highly increased during differentiation of cultured mouse NSCs following growth factor withdrawal (Silber

et al. 2008). MicroRNAs (miR) are small noncoding RNAs consisting of 20–22 nucleotides that participate in gene expression regulation and have emerged as being implicated in the cause of several cancers (Natsume et al. 2011). Although still controversial, miR-21 has been suggested as one of the most commonly upregulated miRs in gloma, and its downregulation by interferon-β was indicated to result from the benefits of such therapy in GSCs (Natsume et al. 2011).

Collectively, these data highlight the requirement for such evaluation of GSCs isolated by different markers to identify potentially unique functional characteristics such as invasive potential and therapeutic resistance. A function has been identified for L1CAM and integrin α_6, for instance: knockdown or inhibition using a blocking antibody reduced tumor growth in a xenograft model (Bao et al. 2008; Lathia et al. 2010). A wide-ranging study increasing and associating marker expression with functional characteristics might reinforce knowledge of the GSC phenotype.

16.1.2 GSC Niche

Stem cell populations are enriched in precise anatomical positions (subventricular zone of the forebrain lateral ventricles, and the subgranular zone in the dentate gyrus of the hippocampus) (Cameron et al. 1993; Weiss et al. 1996) and need a unique microenvironment. It was demonstrated that brain tumor cells that express stem cell markers are found in a perivascular niche and that endothelial cells enhance brain tumor stem cell survival (Calabrese et al. 2007), offering crucial signals (Mirzadeh et al. 2008; Shen et al. 2008; Tavazoie et al. 2008) to maintain self-renewal and an undifferentiated state. Niches present heterogeneous populations including stem cells and surrounding differentiated cells that control essential factors indispensable for determining stem cell fate. These essential factors include stromal supporting cells, soluble factors, ECM proteins, and blood vessels (Jones and Wagers 2008). Endothelial cells are a fundamental element of the GSC niche, also called the "vascular niche" (Shen et al. 2004; Ramirez-Castillejo et al. 2006; Gilbertson and Rich, 2007; Yang and Wechsler-Reya 2007). They secrete paracrine factors that promote stem cell survival and self-renewal.

The interaction of GSCs with blood vessels, and more specifically with endothelial cells and vascular endothelial growth factor (VEGF), became a particular area of interest (Knizetova et al. 2008). Recently, it was demonstrated that $CD133^+$ cells are capable of undergoing differentiation along the endothelial lineage and that a subpopulation of endothelial cells within GBMs are directly derived from tumor cells (Ricci-Vitiani et al. 2010; Wang et al. 2010). Thus, association between endothelial cells and GSCs supports the hypothesis of reciprocal interactions between these two cell populations (Mannino and Chalmers 2011). Surrounding the vasculature are non-neoplastic astrocytes that appear to become reactive to the glioma microenvironment and secrete a number of factors which influence tumor biology (Charles et al. 2011).

The spatial association of GSCs with specific ECM components may influence GSC survival. Three properties have been indicated: the ability of heparin sulfate

(a component of the basement membrane) to bind basic fibroblast growth factor , thus stimulating growth (Loilome et al. 2009); the role of integrins in promoting GBM adhesion, migration, and angiogenesis and the overexpression of the selective $\alpha_V\beta_3$ integrin (Tabatabai et al. 2010); and the overexpression of tenascin C, which stimulates tumor cell proliferation (Midwood and Orend 2009) and inversely correlates with the degree of cell differentiation (Higuchi et al. 1993).

GBMs contain high levels of infiltrated microglia, owing to the local production of chemoattractant factors, which were shown to provide an immunosuppressive environment, contributing to tumor progression (Dimov et al. 2011). Moreover, microglia, by producing metalloproteinases, facilitate the proliferation and migration of tumor cells, as well as the invasion in the brain parenchyma (Alves et al. 2011). Recently, it was demonstrated that GSCs contribute to the conversion of peripheral blood monocytes to immunosuppressive macrophages/microglia, which also exhibit reduced phagocytosis (Wu et al. 2010). Ultimately, it was hypothesized that macrophages could fuse with tumor cells, accounting for their cellular heterogeneity and their invasive properties (Huysentruyt et al. 2011).

Recently, it was suggested that GSCs reside preferentially in the so-called hypoxic niche in the core of the tumor mass. This model of three concentric layers argues that the more differentiated cells are distributed along the peripheral and vascularized part of the tumor (Persano et al. 2011).

In any case, a pronounced time lag between initial mutations and lesion is followed by shorter periods as the tumorigenic clones become more aberrant (the snowball effect), implying that the best therapeutic opportunities are in the early stages, although detection of tumors is difficult in this silent phase (Siebzehnrubl et al. 2011).

16.1.3 GSCs and Tumor Vasculature

Abundant angiogenesis is one of the characteristics of malignant gliomas (Furnari et al. 2007) and an additional mechanism by which GSCs increase tumor growth. Actually, even though angiogenesis is based on the development of new capillaries from preexisting vessels, tumor vasculogenesis is characterized by the recruitment of endothelial precursor cells or bone-marrow-derived hematopoietic cells to form new capillaries. Tumor expansion is restricted by limitations of the supportive vasculature to nourish the tumor and remove products of its metabolism. Initial tumor growth arises from vessel cooption, but ultimately neoangiogenesis is necessary, although the vessels created are abnormal and frequently unproductive. The level of vascularization is considerably associated with the glioma malignancy, tumor aggressiveness, and clinical prognosis (Plate and Risau 1995). On the basis of this knowledge, several groups have investigated the relationship between the tumor vasculature and GSCs. By comparison of in vitro and in vivo development of GSCs in relation to matched nonstem glioma cells, it was determined that GSCs produced tumors with better vascularity than the nonstem tumor cells (Bao et al.

2006b). The mechanism of this hypervascularity was described as involving a secreted factor from GSCs and was shown to induce microvascular endothelial cell migration and vessel construction. Conditioned GSC media were analyzed for the expression of a series of angiogenesis regulators, and elevated levels of VEGF were among the most consistent reports. Another possible explanation for the chaotic vascular organization, besides the overexpression of VEGF, is the poor pericyte recruitment (Bergers and Benjamin 2003). Later, it was shown in a C6 cell line model that GSCs promote both tumor angiogenesis and vasculogenesis via VEGF and stromal-cell-derived factor 1 (SDF-1; Folkins et al. 2009). Thus, it is not surprising that the implementation of bevacizumab to target the effects of VEGF efficiently reduced the GSC proangiogenic properties both in vitro and in vivo. To uncover the upstream mechanisms that induce VEGF expression in GSCs, the function of hypoxia and hypoxia-inducible factors (HIFs) was investigated. As estimated, hypoxia treatment stimulates VEGF expression in both GSCs and non-stem glioma cells, but the levels were always higher in GSCs (Bao et al. 2006b; Li et al. 2009). HIF-1α and HIF-2α exclusively influenced VEGF expression in GSCs in a nonredundant manner. Hypoxia can also increase the GSC population and regulate stem cell marker expression (Heddleston et al. 2009; McCord et al. 2009; Soeda et al. 2009). Therefore, hypoxia may act in tumor progression and therapeutic resistance through its induction of a CSC phenotype.

As glioma cells presenting the CSC markers CD133, HIF-2α, or L1CAM (CD171) are situated near blood vessels (Bao et al. 2006a, b; Li et al. 2009), the symbiotic correlation between GSCs and vasculature may explain the effectiveness of antiangiogenic therapy with bevacizumab (Vredenburgh et al. 2007a, b; Friedman et al. 2009) or cediranib (AZD2171, a VEGF receptor inhibitor) for GBM patients in clinical trials (Batchelor et al. 2007). Indeed, therapeutic application of the anti-VEGF antibody bevacizumab (Avastin) led to reduction of the tumor mass in xenograft models (Bao et al. 2006b; Calabrese et al. 2007). In addition, tumors treated with bevacizumab presented lower levels of CSCs, and cotransplantation of GSCs with endothelial cells led to faster tumor initiation and development (Calabrese et al. 2007). Ultimately, the confirmed efficacy of bevacizumab in recurrent GBM resulted its recent authorization by the FDA, and it is currently being used in clinical trials (Chamberlain 2010).

Additional studies propose that GBM cells can incorporate themselves into tumor vasculature (Shaifer et al. 2010) and direct differentiation of GSCs into tumor endothelium, followed by reduced tumor growth when GSC-derived endothelial cells are directly targeted (Ricci-Vitiani et al. 2010; Wang et al. 2010). It is probable that antiangiogenic therapies might not only reduce tumor vascularization to inhibit GBM growth, but also directly eliminate the niches for the maintenance of GSCs. Thus, inhibition of vasculogenesis is adopted as a new treatment to avoid recurrence (Kioi et al. 2010). One advantage of radiation would be to damage tumor vasculature, thus rendering it more dependent on vasculogenesis to restart development. However, GSCs are radioresistant. This property was investigated via an intracranial GBM xenograft model where it was confirmed that, following radiation treatment, there was a cascade of events that induced

vasculogenesis. Hypoxia in the tumor was the starting point of this cascade, increasing HIF-1 expression, which led to increased levels of SDF-1, which activates the chemokine receptor CXCR4. This phenomenon induces the recruitment of CD11b1 myelomonocytes to the tumor to generate new blood vessels. This mechanism was inhibited by the FDA-approved drug AMD3100, which blocks the interaction of SDF-1 with CXCR4 (Broxmeyer et al. 2005). As GSCs present a radioresistant phenotype, it would be of considerable therapeutic significance to further describe the role of GSCs in postirradiation vasculogenesis. Nevertheless, results from gliomas support an alternative or complementary theory defending the proposal that radiation responses are determined by the "microenvironment stem cell unit" (Mannino and Chalmers 2011).

16.2 Therapeutic Resistance

16.2.1 Multidrug Resistance Phenotypes in Gliomas

The discovery of GSCs identified a new cellular target that might be amenable to novel or traditional treatments. The local recurrence of tumors after surgical resection, radiotherapy, and chemotherapy has pointed to the existence of a population of cells within GBMs that are resistant to treatment. The increasingly popular idea that the chemoresistant and radioresistant GSCs are the cause of GBM recurrence was suggested after the proposal that a CSC is a prerequisite for tumor formation. Correspondingly, there are data supporting the theory that GSCs are resistant to current glioma therapies (Bao et al. 2006a; Liu et al. 2006; Kang and Kang 2007; Nakai et al. 2009; Patru et al. 2010). Intrinsic mechanisms shared with normal stem cells, such as quiescence, self-renewal ability, asymmetric division, long-term proliferation, and multidrug resistance (MDR), allow these malignant stem cells to drive tumor growth and to evade conventional therapy. Normal stem cells have a high proliferative capacity, but they can assume a quiescent state that is known to be closely regulated by the stem cell niche. In fact, slow-cycling stem cells experience reduced chemotherapy- and radiotherapy-induced DNA damage owing to the low accumulation of DNA replication errors. Although there is only limited research confirming that GSCs exist in a quiescent state, it is a commonly cited mechanism for therapy resistance in many tumor types (Schmidt et al. 2001; Naumov et al. 2003; Mellor et al. 2005).

Furthermore, genes involved in DNA repair, such as *MGMT*, and antiapoptotic genes, such as *FLIP*, *BCL2*, *BCLXL*, and *TP53*, were described as being upregulated in GSCs and as important modulators of chemoresistance (Esteller et al. 2000; Liu et al. 2006; Roos et al. 2007; Blough et al. 2010). In addition, GSCs are more efficient than normal stem cells in repairing damaged DNA . Bao et al. (2006a) have shown that DNA checkpoint responses mediated by Rad15, ATM, Chk1, and Chk2 were preferentially activated in the GSC population after

exposure to ionizing radiation. This suggests that GSCs are radioresistant owing, in part, to their enhanced DNA repair ability. Moreover, the capacity of tumor cells to become immortal is one of the main characteristics that distinguishes them from their normal counterparts, and the reactivation of telomerase has been shown to be the most prominent feature of cancer cells. Telomerase activity is a strong indicator of cellular malignancy (Shay and Wright 2002), and in GBM, high levels of telomerase expression correlate with tumor progression and poor prognosis. Recently, Castelo-Branco et al. (2011) have reported that telomerase activity is not a property of all tumor cells, but is rather confined to the GSC subpopulation and is not detectable in most tumor cells.

More recently, tumor cells have been found to be in a state of redox imbalance in a more oxidizing intracellular environment, and show an increased ability to withstand oxidative stress (Ogasawara and Zhang 2009). Thus, malignant cells are less vulnerable to the disturbance of the redox balance generated by xenobiotics. Accordingly, a higher expression of genes related to the metabolism of reactive oxygen species (ROS) was found in brain tumor xenografts (Jamal et al. 2010). Although relatively scarce research has been devoted to the evaluation of ROS levels in CSCs and the redox state in general, as normal stem cells they also exhibit lower intracellular ROS contents than non-CSCs. This may be explained by the increased

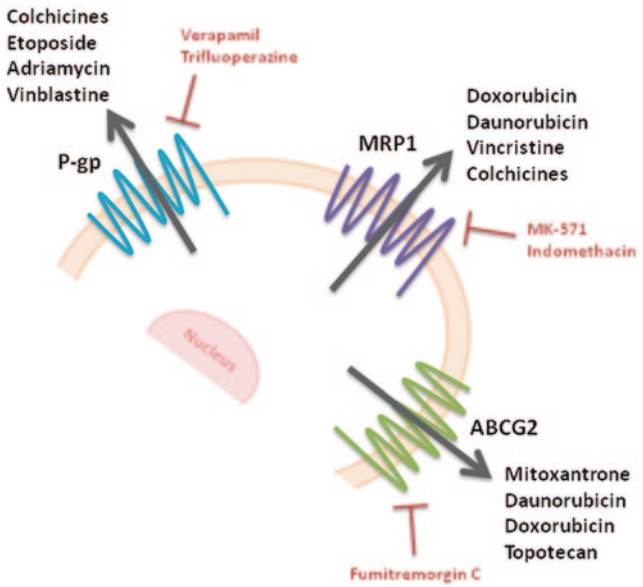

Fig. 16.2 Multidrug resistance (MDR) phenotype in glioma cells is associated with overexpression of ATP-binding cassette transporters. Increased expression of P-glycoprotein (*P-gp*), MDR-associated protein 1 (*MRP1*), and breast cancer resistance protein (ABCG2) in glioma cells is responsible for resistance to multiple chemotherapeutic agents. Several inhibitors (shown in *red*) have been used to study the activity of each transporter

expression of free radical scavenging systems, which may contribute to tumor resistance (Diehn et al. 2009). However, ROS intracellular levels in GSCs remain to be explored. The increased levels of endogenous ROS in tumor cells lead to adaptive changes and may play pivotal roles in tumorigenesis, metastasis, and resistance to radiotherapy and chemotherapy. Several authors have already hypothesized that the high drug resistance of CSCs can be explained by the use of their redox-regulatory mechanisms to evade cell death caused by several anticancer agents. Among the adaptive mechanisms developed by CSCs to survive in this hostile environment are, for instance, the activation of redox-sensitive transcription factors, such as SirT, and the increased expression of antioxidant enzymes, such as glutathione *S*-transferase. Recently, it was demonstrated that GSCs express high levels of SirT1 compared with nonstem tumor cells, and the knockdown of SirT1 expression in these cells was shown to enhance their radiosensitivity (Chang et al. 2009). Furthermore, upregulation of glutathione *S*-transferase expression was observed in many tumor types as well as in gliomas, where it was associated with therapy resistance (Calatozzolo et al. 2005; Fruehauf et al. 2006; Singh et al. 2010). Nevertheless, these redox-regulatory mechanisms in GBM and in GSCs are poorly understood.

Another feature that normal stem cells share with GSCs is increased expression of drug efflux pumps, a well-established cause of MDR. ATP-binding cassette (ABC) drug transporters are expressed in all cells, including GSCs, and are responsible for the efflux of many endobiotics and xenobiotics, such as chemotherapeutic agents (Fig. 16.2). Thus, the tumor recurrence might be related to the inability of cytotoxic compounds to achieve therapeutic concentrations inside CSCs able to eliminate these cells. The high expression of these MDR transporters is one of the most intensely studied and closely associated causes of therapy resistance in gliomas. This is why will return to this subject later.

Along with all of these intrinsic GSC features that promote therapeutic resistance and cell survival after treatments, extrinsic features also seem to play a role in the modulation of therapy response. Direct cell–cell interactions mediated by chemokines, local secretion of cytokines, such as IL-6 and SDF-1, and microenvironmental factors, such as hypoxia, all constitute mechanisms of environmentally mediated chemoresistance (Meads et al. 2008, 2009). Although these mechanisms may substantially contribute to chemoresistance of GBM, there is only indirect evidence to suggest that they may be relevant for GSCs. Further studies are needed to clarify this issue.

16.2.2 MDR Phenotype in Gliomas and the ABC Transporter Superfamily

16.2.2.1 ABC Transporter Superfamily

A well-established cause of the MDR phenotype in tumors, including gliomas, is related to drug transporters, such as the overexpression of members of the ABC

transport superfamily, which is responsible for maintaining low intracellular drug concentrations. The ABC superfamily comprises ATP-dependent efflux pumps and is one of the largest protein families that share sequence and structural homology. These pumps are located in the biological membranes of almost all cells and are responsible for active translocation of a range of substrate molecules through them, such as lipids and other metabolic products, but also fluorescent dyes and drugs. So far, 48 human ABC genes have been identified and have been divided into seven distinct subfamilies (ABCA–ABCG) on the basis of their sequence homology and domain organization. ABC transporters may be full-length transporters, with either two identical halves of nucleotide-binding domains or transmembrane domains, or a half-transporter (with one nucleotide-binding domain and one transmembrane domain) functioning in conjunction with another half-transporter as homodimers or heterodimers (Bunting 2002; Biemans-Oldehinkel et al. 2006). Although the mechanism of ABC transporters is not yet fully understood, proteins associated with the MDR phenotype in tumors, such as P-glycoprotein (P-gp/ABCB1), the MDR-associated proteins (MRPs/ABCCs), and breast cancer resistance protein 1 (BCRP1/ABCG2), have received considerable attention in studies of chemoresistance (Fig. 16.2).

16.2.2.2 ABC Transporters in Gliomas

The MDR phenotype mediated by the members of the ABC transporter superfamily is recognized in the literature as one of the most significant mechanisms of chemoresistance, and a vital one in tumors, including gliomas. Thus far, the most relevant glioma-related ABC transporters are P-gp, MRPs (especially MRP1), and ABCG2.

P-gp was the first ABC transporter to be described and, as an ATP-dependent drug efflux pump, it has been demonstrated to transport various substrates comprising hydrophobic substrates/drugs as well as steroids, lipids, and peptides (Dean et al. 2001; Gottesman et al. 2002). P-gp plays a number of physiological roles in a variety of normal tissues, including the blood–brain barrier and many secretory cells such as those in the kidney and liver. Moreover, it is also expressed in many malignant tumors and can actively protect tumor cells from high levels of chemotherapeutics. Actually, the MDR in tumor cell lines was originally attributed to the overexpression of P-gp more than a decade ago (Ambudkar et al. 1999). Since then, numerous studies have been published regarding the overexpression of this transporter and its association with therapeutic resistance in gliomas. Roninson et al. (1986) provided the first evidence of the involvement of P-gp in drug resistance, showing that the human gene *MDR1* alone, responsible for encoding P-gp, could transfer the drug-resistant phenotype to drug-sensitive cells. The expression level of *MDR1* was reported to be increased in high-grade gliomas, including GBMs, compared to low-grade astrocytomas (Spiegl-Kreinecker et al. 2002). Also, low levels of P-gp expression were detected in cells derived from normal human asctrocytes, suggesting that *MDR1* expression may be linked to the degree of

malignancy of astrocytomas (Bahr et al. 2003). It was also demonstrated that P-gp expression increases in patients examined after treatment, implying that P-gp may be responsible for MDR in gliomas, even though there were only 4–23 % P-gp-positive cells in human gliomas before chemotherapy (Abe et al. 1998). In contrast, Mousseau et al. (1993) have shown that P-gp overexpression was detected in only 2 % of 67 human primary and metastatic brain tumors, including 31 gliomas. Moreover, overexpression of P-gp was found to be relatively rare in glioma cells compared with the high expression of MRP1 in normal astrocytes and glioma cells. This suggests that it may be MRP1 rather than P-gp that contributes to the intrinsic chemoresistance in gliomas (Spiegl-Kreinecker et al. 2002). Nevertheless, even with conflicting data, the role of P-gp expression as a prognostic marker appears to be widely accepted and has been proved in a series of studies of malignant tumors, such as neuroblastomas, childhood medulloblastomas, ependymomas, and gliomas (Chan et al. 1991; Chou et al. 1995; Korshunov et al. 1999). Studies in glioma cells using P-gp inhibitors showed enhanced cytotoxic effects of chemotherapeutic agents in the absence of P-gp activity, reinforcing the important role of P-gp in the mediation of glioma resistance (Bahr et al. 2003). In addition, Korshunov et al. (1999) have shown an improved progression-free survival time and recurrence-free survival time in P-gp-negative tumor specimens derived from low-grade ependymomas, detected by immunohistochemistry, among 76 patients examined. Overexpression of P-gp was also gound to be correlated with a reduced overall survival rate in patients with low-grade gliomas (Andersson et al. 2004).

MRP1 was first discovered in a human small cell lung cancer cell line (H69AR) selectively resistant to anthracycline (Cole et al. 1992) and it has been extensively associated with MDR in gliomas. It is capable of transporting a wide variety of structurally diverse substrates, such as organic anions, cysteinyl leukotriene C_4, and glutathione, as well as a number of chemotherapeutic agents across the cell membranes of stomach, lung, brain, and cells from most tissues throughout the body (Deeley and Cole 2006). One of the most important physiological functions of MRP1 is to maintain the glutathione intracellular homeostasis, but also to contribute to cell detoxification via rapid efflux of water-soluble conjugates of cytotoxic drugs (Jedlitschky et al. 1994; Deeley and Cole 2006). Because MRP1 was detected at the membrane level of vascular endothelial cells in 14 of 15 high-grade gliomas, it is suggested that MRP1 is responsible for the limitation of uptake of cytotoxic drugs, thus preventing such drugs from reaching a concentration able to trigger cellular toxicity (Benyahia et al. 2004).

Clinical studies have documented the overexpression of MRP1 in a variety of hematological and solid tumors, including gliomas, with a correlation between MRP1 expression and negative response to treatment and disease outcome. These studies have been reviewed extensively elsewhere (Kruh and Belinsky 2003; Andersson et al. 2004; Deeley and Cole 2006). Curiously, Calatozzolo et al. (2005) reported the absence of differences in MRP1 expression with respect to primary and recurrent gliomas. Thus, it is believed that the resistance of glioma cells to chemotherapy is intrinsic. In the same study, high levels of MRP1 expression in 48 glioma specimens and 21 primary cultures were observed, and MRP1 was

shown to be the most frequent ABC transporter in high-grade gliomas. The preferential presence of MRP1 in high-grade instead of low-grade gliomas was also observed by Haga et al. (2001). Studies using indomethacin as an MRP1 inhibitor have shown that its inhibition in glioma cells significantly increased the cytotoxic effect of etoposide, a chemotherapeutic agent used in GBM therapy. This suggests that the association of MRP1 inhibitors with chemotherapy could be of interest in the clinical management of gliomas (Benyahia et al. 2004). Recently, Peignan et al. (2011) have supported those results by using MK571 to inhibit MRP1 activity. Altogether, these data point to a significant role of MRP1 in clinical drug resistance, thus emerging as an attractive therapeutic target.

Moreover, other members of the MRP family were identified as overexpressed in gliomas and consequently emerged in the chemoresistance scenario in these tumors. MRP3 (cMOAT2 or ABCC3) is overexpressed in some tumor cell lines that have acquired MDR (Kool et al. 1997; Uchiumi et al. 1998) and is also highly expressed in GBMs, but not in normal brain cells (Loging et al. 2000). Nevertheless, Calatozzolo et al. (2005) showed heterogeneous data regarding MRP3 expression in different glioma specimens by revealing it to be more commonly present in immunohistochemical sections of high-grade gliomas, but evidenced a low protein expression by flow cytometry in overall glioma specimens, contrasting with high messenger RNA (mRNA) levels detected by quantitative real-time PCR. Anyway, MRP3 was also related to the chemoresistance phenomenon since it showed capacity to modulate drug sensitivity to certain anticancer agents in human gliomas (Haga et al. 2001). Recently, in a screening of MRP3 mRNA expression in GBM samples, upregulated mRNA levels of this transporter were shown in most of the GBM cases, and glioma cells expressed MRP3 protein both in vitro and in vivo (Kuan et al. 2010). In the same study, MRP3 mRNA expression in GBM biopsies was shown to be correlated with a higher risk of death.

Bronger et al. (2005) have also detected the expression of MRP4 and MRP5 in glioma cells (Decleves et al. 2002). MRP5 was found to be moderately expressed in GBM (Alexiou et al. 2012) and was mostly found in high-grade gliomas (Calatozzolo et al. 2005). MRP4 and MRP5 were detected by RT-PCR in two distinct GBM cell lines (GL15 and 8MG) and were also demonstrated to be implicated in an intracellular decrease of vincristine and etoposide concentrations (Decleves et al. 2002). These membrane transporters were also found to be expressed in human glioma specimens and to be associated with an astrocytic phenotype (Bronger et al. 2005). In accordance, the highest levels of MRP5 were also encountered in astrocytes and endothelial cells either in primary or in recurrent gliomas (Calatozzolo et al. 2005). In rat C6 glioma cells, the relative expression of MRPs was as follows: MRP4 > MRP1 > MRP5 > MRP3 > MRP2 (Decleves et al. 2008).

Finally, ABCG2 is a half-transporter that plays an important role in conferring the MDR phenotype (Doyle et al. 1998) owing to its capacity of effluxing different cytotoxic drugs. ABCG2 has been found in normal tissues such as placenta, liver, and intestine, as well as in a variety of tumor-resistant cells, including

breast, colon, lung, and ovarian cancers. The overexpression of ABCG2 may or may not be correlated with P-gp and MRP1 expression in some of these malignancies (Bates et al. 2001; Doyle and Ross 2003). ABCG2 has been detected in microvessel endothelial human brain and glioma cells, implying that it may play a crucial role in both normal brain function and glioma treatment by modulating the transport of drugs into the brain (Bleau et al. 2009b; Valera et al. 2009). ABCG2 may also be used as a predictor for the outcome of glioma treatment, although at present there is insufficient evidence to support this statement (Lu and Shervington 2008). Human brain vessels and parenchymal tissue extracted from GBM displayed higher levels of ABCG2 than normal brain tissue (Zhang et al. 2003; Aronica et al. 2005; Bleau et al. 2009b; Bhatia et al. 2012).

16.2.2.3 ABC Transporters and GSCs

GSCs have been extensively associated with therapeutic resistance, and have been described as being responsible for GBM recurrence, owing to their stronger MDR phenotype. In recent years, it was conceptually accepted that one of the main characteristics of NSCs and CSCs is increased expression of ABC transporters compared with nonstem cells (Hirschmann-Jax et al. 2004; Schatton et al. 2008). This suggests that targeting these drug transporters may reduce the chemoresistance of the CSC population in tumors. Also, GSCs have been found to correlate with high expression of ABC transporters, mainly P-gp, MRP1, and ABCG2.

In several studies, P-gp expression was reported to be increased in GSCs. Nakai et al. (2009) have shown an increase of *MDR1* expression in a stronger drug-resistance GSC line as compared with a glioma cell line, and cross-reactivity of the GSC marker CD133 and P-gp in a surgical specimen of GBM. Moreover, P-gp was found not only to be upregulated in GSCs, but also to exhibit increased activity, thus preventing the cellular entry of cytotoxic compounds (Angelastro and Lame 2010).

Despite the higher expression of P-gp in GSCs, MRP1 has recently received more attention owing to its higher levels in GSCs (Salmaggi et al. 2006; Jin et al. 2008; Shervington and Lu 2008; Peignan et al. 2011). Curiously, in opposition to the significant increase of the MRP1 mRNA expression in GSCs, a downregulation was observed when GSC differentiation is induced. Also contributing to the chemoresistance is the substantial increase of MRP1 expression in GSCs that occurs after etoposide exposure (Jin et al. 2010).

ABCG2 is associated with the MDR phenotype of GSCs as well and is responsible for the effective reduction of the intracellular concentrations of several prominent anticancer chemotherapeutic agents (An and Ongkeko 2009; Bleau et al. 2009a). So, modulation of ABCG2 expression may be a promising therapeutic strategy. Preliminary data have shown that the downregulation of ABCG2 in GSCs, using miR-328, may be a possible mechanism to decrease chemoresistance in these cells (Li et al. 2010). In addition, since ABCG2 can transport a range of different dyes in both nonstem and stem-like cells, and the $ABCG2^{+}$ subset of

tumor cells is often enriched in cells with a CSC-like phenotype, ABCG2 has also been used to define the undifferentiated SP cells (An and Ongkeko 2009; Bleau et al. 2009a). The resultant ABCG2$^+$ cells were found to be highly expressed in the SP tumor cells (Zhou et al. 2001; Hirschmann-Jax et al. 2004; Challen and Little 2006). Hirschmann-Jax et al. (2004) found a distinct SP in two glioma cell lines and in neuroblastoma samples expressing high levels of ABCG2. This SP of neuroblastomas was isolated and proved to have the capacity to divide asymmetrically, generating SP and non-SP cells, and to induce greater chemoresistance. It has been suggested, therefore, that potential therapeutic strategies can be devised to target particularly the SP because of its association with intrinsic resistance to chemotherapy (Lu and Shervington 2008).

With all these conflicting data and a lack of information about the role of ABC transporters in GSCs, further studies must be conducted to disclose the pattern of ABC transporters in these malignant cells and make them promising and new potential targets for glioma therapy.

16.3 GSC as a Target for Tumor Therapy

Directed target drugs that competently eradicate CSCs might be a more successful in treating GBM. GSCs are, therefore, a new cellular target to be focused on in novel treatments.

The establishment of GSCs in vitro requires the addition of EGF to the culture medium (Lee et al. 2006), revealing the importance of this signaling pathway for the survival of these cells. Recent studies demonstrated that EGFR kinase inhibitors dramatically reduce the proliferation and self-renewal levels of these cells, increasing apoptosis of the CD133$^+$ population (Soeda et al. 2008). The EGFR inhibitors erlotinib and gefitinib have been clinically adopted for the treatment of brain tumors, even though more than 80 % of patients had no response to these agents (Rich et al. 2004; Prados et al. 2006, 2009). GBMs usually present a mutated or deleted phosphatase and tensin homologue (PTEN), a tumor suppressor that inhibits the phosphatidylinositol 3-kinase pathway, which, by being a downstream event in variant EGFR signaling (Yuan and Cantley 2008), possibly explains the lack of response of GBM to EGFR inhibitors. PTEN negatively regulates NSC proliferation by inhibiting mammalian target of rapamycin (mTOR) through the phosphatidylinositol 3-kinase pathway (Groszer et al. 2001). Loss of *PTEN*, frequently seen in GBM, leads to mTOR activation, therefore modulating the translation of a range of proteins that are essential for cell cycle progression.

Clinical analysis of sirolimus (or rapamycin), an inhibitor of mTOR complex 1, and CCI-779, a derivative of sirolimus that induces cell cycle arrest in G1 phase, has been performed in GBM patients. All studies revealed the need for a combined therapy to achieve a meaningful clinical outcome (Chang et al. 2005; Galanis et al. 2005; Doherty et al. 2006; Cloughesy et al. 2008; Reardon et al. 2010).

Viral vectors have been adopted in clinical trials to transmit therapeutic genes into cancer cells to target the genetic alterations present in GBM. As there is a high level of overlap between the signaling pathways, it is useless to target individual genes as an antitumor strategy. Furthermore, replication-deficient viral vectors cannot transfect enough cancer cells, being ineffective as an GBM therapy. Therefore, studies are focused on advances to target common pathways in cancer cells, seeking the improvement of drug cytotoxicty. The replication-competent oncolytic adenovirus Delta-24-RGD may achieve that goal, as it is not focused on targeting a single molecule or pathway. Otherwise, Delta-24-RGD is capable of engaging the complete cellular system within a cancer cell for its own replication (Berk 2007). Although CSCs are resistant to conventional therapies, brain tumor stem cells are susceptible to adenovirus-mediated cell death via autophaghy in vitro and in vivo. After infecting the CSCs, Delta-24-RGD leads to the generation of autophagic vacuoles in the cytoplasm, followed by cell lysis (Jiang et al. 2007). The defective retinoblastoma pathway allows Delta-24-RGD to replicate and to selectively abolish CSCs (Jiang et al. 2007). As Atg5, an essential protein in the autophagic apparatus, was upregulated at the late stage of viral infection, it was assumed that the infected cells suffered autophagy, followed by the release of viral progenies. The survival time of GSC-xenotransplanted mice was significantly improved when they were infected with Delta-24-RGD (Jiang et al. 2007). After the infection, an upregulation of Atg5 in the tumor was noticed as well, suggesting that the Atg5 level can be adopted as a marker to monitor Delta-24-RGD therapy in clinical trials. A phase I clinical trial to estimate the usefulness of Delta-24-RGD in patients with recurrent GBM is under way (Anderson 2008).

Other therapeutic approaches to GSCs result from clinical trials that have strengthened the focus on antiangiogenic therapies after discovery of the perivascular CSC niche. The antiangiogenic drugs bevacizumab (Vredenburgh et al. 2007a, b) and cediranib (AZD2171) (Batchelor et al. 2007) have exhibited high efficiency in patients with GBM. Reduction of the tumor mass could be a result of the eradication of tumor blood supply, a recognized mechanism of antiangiogenic drugs, or could signify that these drugs may also reduce the survival of the GSCs present in the perivascular region (Calabrese et al. 2007). This theory is also reinforced by the discovery that antiangiogenic drugs usually reduce the proliferation or survival of most cancer cells.

Recently, the ability of NSCs to migrate to tumors has raised the possibility of their use as carriers of therapeutic, antineoplastic agents (Ahmed and Lesniak 2011). In addition, the immunosuppressive qualities of NSCs are a very appealing attribute as therapeutic genes express their products for longer and oncolytic viruses replicate for longer and kill tumor cells without significant immune interference. In contrast with methods using antibodies against cancer-cell-specific antigens, NSCs have the ability to cross the blood–brain barrier and the potential to sustain a high therapeutic antibody concentration at the tumor site.

As the origin of CSCs in GBM from each patient may diverge and they may exhibit singular genetic alterations in complex tumor tissues, potential treatment of GBM may depend on an exclusive combination of some targeted therapies,

based on the cellular, genetic, and molecular information on the tumor in the individual patient.

16.4 Future Directions

The assimilation of information from basic and clinical investigations has significantly improved the understanding of GBM biology. The poor survival of patients, however, remains a challenge and more refined tools are required to clarify the details of GBM formation. The boundary between human cancer and genetic mouse models must be completely broken in order to rapidly convert basic science data into practical clinical applications.

In addition, since GSCs may change and differently adapt in situ, selective GSC targeting will be challenging. It must also be clarified how GSCs contribute to recurrence following therapeutic intervention, in order to allow the development of new strategies targeting the elimination of these cells on the primary presentation of the disease. Moreover, it will be fundamental to find out if microglia and stromal cells themselves should be considered as potential targets and to understand whether targeting the tumor vasculature will disrupt the perivascular niche with the loss of support.

Genetic and cellular investigations of GBM cell populations from patients at different stages of disease, at the single-cell level, should offer considerable insight into the relationships between normal cells, the cells of origin, and GSCs. Identification of the cell of origin may allow a more efficient analysis of the genetic defects implicated in GBM initiation and development, and may function as a platform for the identification of early tumor biomarkers. It may also have an essential repercussion in avoiding relapse, mainly in patients in which relapse is a consequence of a "premalignant" clone (possibly the cell of origin itself) that continues in the patient before acquiring a mutation that renders it malignant. If so, even patients with GBM who have intense regression may need maintenance therapy to diminish the possibility of relapse. Finally, the gene signature of the cell of origin may clarify fundamental molecular pathways and mutations that could lead to new therapeutic advances to prevent or target early-stage GBM.

Gene-expression profiling studies, assays that aim to establish novel diagnostics and to establish or to test therapeutics, should be executed on primary GBM cells, rather than GBM cell lines, whenever possible. Markers can become uninformative in culture, as there is definitely a constant selection process that impairs the ability to draw conclusions about hierarchy. Consequently, findings about markers from in vitro culture may not be extended to primary tumors. This becomes more evident when the microenvironment is taken into account. CD133 and other cell surface markers mediate signals between cells and the microenvironment. Therefore, their expression and value in isolation of GSCs may be disregarded if they are assessed in culture versus a freshly dissociated tumor. It is also imperative to take into account the probability of gene-expression differences between tumorigenic cancer cells and cancer cells with restricted proliferative potential. By

doing so, we might be able to reveal previously unrecognized variations between cancer cells that will allow a more efficient classification, diagnosis, and treatment of cancers. A better understanding of the interaction of GSCs with their microenvironment and their influences on immune cells might help in the establishment of novel and effective strategic therapies to combat GBM.

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Chapter 17
The Tumor Microenvironment as a Transient Niche: A Modulator of Epigenetic States and Stem Cell Functions

Lorena E. Mora-Blanco, James B. Lorens and Mark A. LaBarge

Abstract For the cancer stem cell hypothesis to exist, a complimentary "niche" or tumor microenvironment must be present to provide a proper context for the malignant cell to flourish. There are a myriad of factors produced by the tumor microenvironment to regulate tumor initiation, growth, and survival. These signals undoubtedly alter and are altered by epigenetic regulation of gene expression programs that are also tightly linked to both stem cell pluripotency and malignancy. Yet little work has been done to interrogate this interaction. As our understanding of the interplay between malignant cells, their niche microenvironments, and the change of epigenetic states develops, we will gain better ability to generate relevant systems to model these interactions as well as potentially providing rational novel entry points for drug design.

Keywords Tumor microenvironment • Niche • Epigenetic • Stem cells • Cancer

17.1 Introduction

Cancer is described as a disease of development gone awry, the result of normal developmental pathways hijacked by oncogenic processes. The cancer stem cell (CSC) hypothesis states that a subset of immortal cells are endowed with stem-cell-like self-renewal traits to supply growing tumors with more differentiated neoplastic cells (Rosen and Jordan 2009). This offers an attractive explanation

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for the observed cellular heterogeneity within tumors, acquisition of metastatic capability, and ability to resist therapy. The underlying logic of the CSC concept is modeled on normal developmental hierarchies delineated for a number of normal adult tissues. By extension, this necessarily includes the existence of a *CSC niche*. Niches are specialized microenvironments within tissues, wherein stem and progenitor cells reside (reviewed in Fuchs et al. 2004; Scadden 2006). Any microenvironment is defined by the sum of cell–cell, cell–extracellular matrix (ECM), and cell–soluble factor interactions, and physical and geometric constraints that are experienced by a cell. In normal tissues, niche microenvironments exert a dominant influence over stem cell function. Progenitors in both skin and skeletal muscle can adopt residency in vacated stem cell niches and reacquire stem cell traits (Nishimura et al. 2002; Collins et al. 2005; Sacco et al. 2008). Impressively, testis and neural stem cells from male mice transplanted into the mammary fat pad give rise to lactating mammary glands (Boulanger et al. 2007, Booth et al. 2008a). Mimetic combinatorial microenvironments reveal a substantial and quantifiable flexibility of embryonic and adult stem and progenitor cell fate decisions (Flaim et al. 2005; Soen et al. 2006; LaBarge et al. 2009). The ability of the niche, or more broadly defined, the microenvironment, to determine the functional spectrum of stem cell activities led us to hypothesize that stem cell niche microenvironments beget stem cell functions (LaBarge et al. 2007). Thus, it is important to consider how tumor microenvironments may impose stem-cell-like functionality on tumor cells and create CSCs.

The inherent genetic instability of tumor cells and variable host response to neoplasia continuously reshapes the tumor microenvironment (Egeblad et al. 2010). Intuitively, this dynamic landscape comprises microenvironments that elicit stem-like phenotypes in tumor cells necessary to drive malignant progression. Indeed, affecting the tumor microenvironment with targeted therapeutics renders tumor cells more aggressive (Kerbel 2009). Tumor aggressiveness has long been associated with loss of epithelial barrier function (e.g., downregulation of E-cadherin) and acquisition of invasive mesenchymal traits and markers (e.g., vimentin). During normal early embryonic development, epithelial cells acquire transient mobility via an epithelial-to-mesenchymal transition (EMT), which is an epigenetically regulated gene program (Thiery et al. 2009). Importantly, adult stem and progenitor cells express EMT transcription factors (e.g., Snail, Slug, Twist, and Zeb) and aspects of the EMT gene program are important for normal adult tissue homeostasis (Sicklick et al. 2006). Induction of EMT in tumor cells correlates also with the CSC phenotype. For example, immortal mammary epithelial cells exposed to active transforming growth factor β (TGF-β) or overexpressing EMT transcription factors activated stem-cell-like gene expression profiles and elicited malignant cell behavior (Mani et al. 2008). EMT and CSC-gene expression is prevalent in highly aggressive breast cancer subtypes (Karnoub et al. 2007). Microenvironmental effectors that enhance malignancy also involve epigenetic EMT mechanisms. ECM remodeling by metalloproteinases frequently observed in breast cancers contributes to genomic instability and EMT in mammary epithelial cells (Sternlicht et al. 1999; Radisky et al. 2005) and tumor formation in

mice (Sternlicht et al. 2000). Both the composition and the tensile attributes of the ECM upregulate tumor cell EMT-like and CSC-like traits (Kirschmann et al. 2002; Paszek et al. 2005; Levental et al. 2009). This illustrates the concept that a number of known tumor microenvironment components can induce a CSC phenotype, and thus are potential CSC niche constituents.

Epigenetic regulation of gene expression programs through the actions of DNA- and histone-modifying enzymes and small noncoding RNAs is inextricably linked to embryogenesis and stem cell pluripotency. The plasticity of the CSC phenotype is similarly congruent with epigenetic regulation. Epigenetic mechanisms contribute frequently to malignancy; tumor suppressor gene promoters are often downregulated by methylation and expression of epigenetic regulatory proteins is associated with drug resistance (Sharma et al. 2010). In this chapter, we will review how interactions between tumor cells and niche-like microenvironments may elicit epigenetic states that explain emergent stem-cell-like functionality of some cells within tumors.

17.2 Stem Cell Niche Microenvironments in Normal and Malignant Tissues

Microenvironments are defined by the sum of chemical and physical interactions that effect cells: ECM and soluble factors, including growth factors and cytokines, activate cognate cellular receptors and signal transduction; oxygen tension modulates specific gene expression networks; tensile forces modulate signal pathways via cytoskeletal proteins. Niche microenvironments within specific anatomical architectures tightly control stem cell gene regulatory networks. Stem and progenitor cells have the unique ability to both self-maintain and give rise to many types of more differentiated cells that serve specialized functions within a tissue. Within the embryo, embryonic stem cells derived from the inner cell mass of a blastocyst have the ability to give rise to the three embryonic germ layers: ectoderm, mesoderm, and endoderm. As the embryo develops, germ stem cells and adult stem cells are generated for reproduction and organogenesis, respectively. Although not pluripotent as embryonic stem cells are thought to be, adult stem cells are typically associated with a cognate tissue or organ, and they possess the capability to self-maintain. Adult stem cells are more restricted in their potency than are embryonic stem cells; adult stem cells are able to give rise to multiple lineages of a tissue, or are unipotent. However, experimental systems in which adult stem cells were forcibly transplanted into noncognate tissues demonstrated a microenvironment-dependent flexibility of cell fate decision (Nishimura et al. 2002; Boulanger et al. 2007; Booth et al. 2008b). The stem cell niche varies dependent on the type of tissue and location. Schofield (1978) was among the first to discuss the existence of stem cell niches when he hypothesized that physiologically limited microenvironments that support stem cells should exist. Specialized stem cell microenvironments were first defined experimentally in invertebrate model systems. In the

gonads of both *Drosophila melanogaster* and *Caenorhabditis elegans*, the germ cells reside at the distal end of a tapered structure, the germarium in *D. melanogaster* and the distal tip in *C.elegans*, and have been shown to depend on interactions with adjacent somatic cells at the end of this structure to maintain stem cell features (Kiger et al. 2000; Xie and Spradling 2000; Crittenden et al. 2002). In adult mammal tissues, several stem cell niches have been putatively identified, including niches for the stem cells of the hematopoietic, skin, intestinal, breast, and neural compartments (Li and Xie 2005).

Conceptually parallel to the role of adult stem cells in organogenesis and homeostasis is the idea that tumors are established and maintained by CSCs (or tumor-initiating cells). The concept suggests that similarly to developmental hierarchies in which normal stem cells are at the apex, CSCs also sit on an apex of a developmental hierarchy that leads to a tumor. CSCs reputedly possess the capability to divide asymmetrically, generating both another stem cell and a daughter progenitor that has the capacity to generate heterogeneous lineages of neoplastic cells that constitute the bulk of a tumor. Analogous to the stem cell niche, the CSC phenotype is maintained experimentally by specialized microenvironmental conditions, including hypoxia, low pH, TGF-β, and collagen I, conditions that also support tumor proliferation and promote resistance to genotoxic stress and apoptosis. Although the CSC concept is increasingly accepted, quantifying the tumor-initiating ability of CSCs remains challenging as the conditions governing in vivo tumorigenic barriers differ between host strains and malignancies. Many studies have concluded that tumor-initiating CSCs are rare in most human cancers (Ishizawa et al. 2010). However, recent studies that directly tested the host requirements of putative CSCs in human and murine tumors of the skin, colon, and breast also concluded that within one tumor there are multiple, distinct populations of cells that are tumorigenic, and not necessarily rare, and thus do not fulfill the strict hierarchical criterion of stem cells (Quintana et al. 2008; Shmelkov et al. 2008; Ishizawa et al. 2010; Kim et al. 2012). Historically the tumor "stem line" concept also has not held up to scrutiny (Hauschka and Levan 1958; reviewed in LaBarge 2010)). Arguably, tumors are at best a distorted reflection of the cognate organ. Hence, the CSC niche may similarly only comprise a semblance of normalcy. Tumor angiogenesis provides an apt paradigm: normal tissues regulate new blood vessel formation via balanced proangiogenic and antiangiogenic microenvironmental effectors to form well-pruned vascular arborizations scaled to serve metabolic requirements; in contrast, tumor vasculature, although created via the same molecular mechanisms, is immature, tortuous, leaky, and poorly functional, fueling a need for sustained angiogenesis.

The identity and existence of CSCs notwithstanding, a common genetic program is shared between embryonic stem cells, adult stem cells, and CSCs. Gene expression normally associated with EMT has been identified in normal stem cells and CSCs, and may be required by the CSCs to metastasize and establish tumors (Yang et al. 2004; Mani et al. 2008; Guo et al. 2012). Ectopic expression of EMT transcription factors imposes CSC-like functions in many model systems (Thomson et al. 2011). These EMT transcription factors alter epithelial gene expression profiles, demarcated by the repression of genes encoding apical–basal

polarity, epithelial junctional complexes (E-cadherin), and cytokeratins, and induce the expression of the mesenchymal proteins vimentin and N-cadherin, resulting in increased motility and invasiveness. When in the mesenchymal state, tumor cells display enhanced tumor-initiation and metastatic activity. Microenvironmental activators of EMT similarly create CSCs in many systems. Early pancreatic carcinomas harboring oncogenic mutations (Ras) undergo EMT as the result of local inflammatory stromal signals (Rhim et al. 2012). EMT transcription factor activity is promoted by inflammatory mediators (Wu et al. 2009). The hypoxic tumor microenvironment, known to enhance tumor aggressiveness, induces EMT and CSC activity (Yang et al. 2008; Storci et al. 2010; Cooke et al. 2012). EMT states are stabilized by different ECM proteins (Kirkland 2009). Other common tumor microenvironment components such as TGF-β are known inducers of EMT programs and could be involved in maintenance or induction of EMT-like phenotypes in cells within tumors (Wendt et al. 2009). Moreover, tumor-associated hepatocyte growth factor producing fibroblasts were able to dedifferentiate nontumorigenic cancer cells into more immature phenotype by reactivation of the Wnt pathway in a colon cancer model (Vermeulen et al. 2010). The dedifferentiated cells exhibit characteristics of CSCs, which include expression of stem-cell-associated genes and increased tumorigenic potential. Indeed, although we have argued that CSC niches are nearly impossible to identify (LaBarge 2010), there is robust experimental evidence that specific microenvironmental components of tumors can impose quiescent states upon some tumor cells that make them less susceptible to cancer therapeutics, which are thought to be an important component of cancer recurrences and minimal residual diseases (Matsunaga et al. 2003). Given the unstable nature of tumors and the plasticity of the CSC phenotype, one could reasonably assume that CSC niches are transient constellations lacking strict anatomical definition that are difficult to predict.

Because tumors develop over long periods of time and recurrent tumors remain dormant for many years, there may be a means for maintaining the stem-like program in a subset of tumor cells for protracted periods. Genetic mutations incurred during tumorigenesis may set the stage for acquiring stem-cell-like functions, but the observation that different populations in the tumor can regenerate the tumor with differing efficiencies suggests that epigenetic regulation, a more flexible and heritable form of regulation, may also be at work. Indeed, melanomas can form from tumor cells that express or do not express the CSC marker CD133, and reestablish the original ratios of $CD133^-$ to $CD133^+$ cells; 15 commonly used CSC markers behaved similarly to CD133 (Quintana et al. 2008, 2010; Shackleton et al. 2009). The results of these experiments indicated that individual cancer cells were capable of recapitulating the marker heterogeneity of the tumors from which they are derived. These results support the notion that neoplastic cells toggle between CSC and non-CSC states via epigenetic mechanisms (Gupta et al. 2011; Scheel and Weinberg 2011). Subpopulations of human melanoma cells in culture differentially express the histone H3K4 demethylase JARID1B, an epigenetic regulatory enzyme (Roesch et al. 2010). Further, whereas the $JARID1B^+$ subpopulations cycle more slowly than $JARID1B^-$ cells, the $JARID1B^+$ tumor cells generate

more progeny and are more tumorigenic. These results suggested that the expression of epigenetic regulatory proteins such as JARID1B has a vital role in the CSC state of tumor cells. Relatedly, expression of the JARID1A H3K4 histone demethylase in a subpopulation of reversibly drug-tolerant cells showed a greater than 100-fold reduced drug sensitivity (Sharma et al. 2010). The drug-tolerant/drug-resistant phenotype was transiently acquired and lost at low levels in individual cells. Together these data suggested a critical role for epigenetics in the acquisition and maintenance of CSC properties (Fig. 17.1).

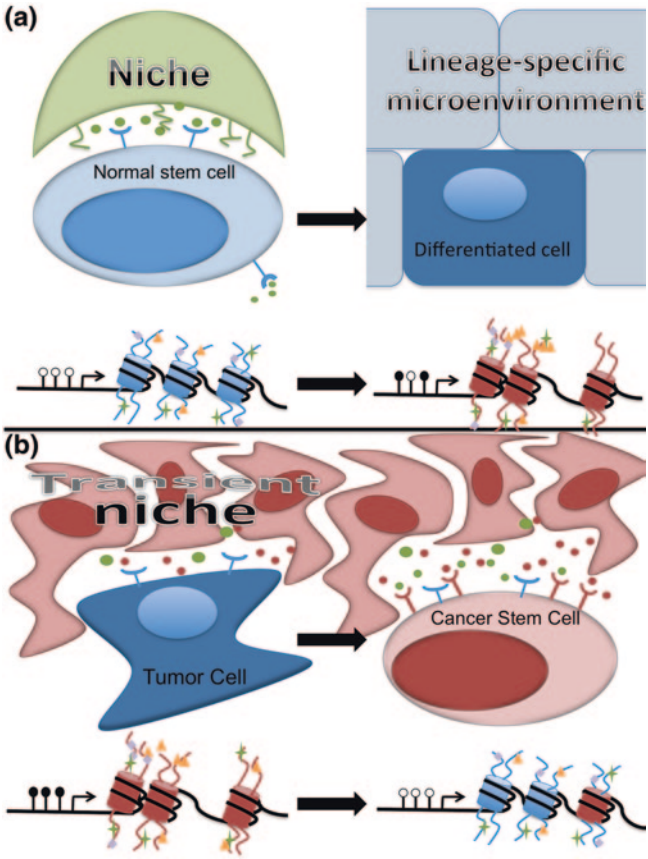

Fig. 17.1 Control of epigenetic modifications by the microenvironment. **a** In the normal context, stem cells are regulated by their normal microenvironment, the stem cell niche, where they have distinct epigenetic patterns. These cells, through additional cues, differentiate into normal somatic cells and in doing so change their epigenetic state. This state is maintained, in part, by their lineage-specific microenvironment, and includes DNA methylation (*closed circles* at the promotor), histone modification (*triangles*, *crosses*, and *diamonds*) on histone tails, and nucleosome position (microRNAs not shown). **b** A tumor cell is guided by transient tumorigenic niches that alter their epigenetic state to that of a more undifferentiated cell, becoming a cancer stem cell

17.3 Establishing Links Between Epigenetic States and Tumor Microenvironments

It is increasingly clear that genetic mutations are not the singular path to gene disruption in cancer, and a critical role for epigenetic changes in tumorigenesis is well understood. The prevalence of epigenetic changes caused by the tumor microenvironment, however, is still emerging. Epigenetics is broadly defined as heritable changes in gene expression or cellular memory not encoded by the underlying DNA sequence. By this definition, microenvironmental influence could also be considered an epigenetic phenomenon, but generally the term is limited to traits intrinsic, rather than extrinsic, to the cell. Here, we focus on the epigenetic phenomenon of DNA methylation, chromatin remodeling and histone modification, and microRNAs (miRNAs), and the likelihood that these states are imposed by components of the tumor microenvironment. We discuss the links that have been made between the interplay of epigenetics and the microenvironment in the malignant context.

17.3.1 DNA Methylation

DNA methylation involves the addition of a methyl group at position 5 of the cytosine pyrimidine ring of DNA. In mammals, DNA methylation is critical to normal development and is involved in the key processes, such as X inactivation, genomic imprinting, and repetitive element suppression. The function of DNA methylation is to repress transcription, by inhibition of transcription factor binding and via recruitment of corepressor complexes. Two distinct enzymatic processes carry out DNA methylation: de novo methylation and maintenance methylation. De novo methylation is catalyzed by DNMT3A and DNMT3B methyltransferases, whereas maintenance methylation is performed by DNMT1. Their critical role in development is highlighted by the fact that gene knockout of either *DNMT1* or *DNMT3* is not viable (Li et al. 1992; Okano et al. 1999). Aberrant DNA methylation was first linked to cancer nearly three decades ago with the observation that the genomes of cancer cells are globally hypomethylated compared with their normal counterparts (Feinberg and Vogelstein 1983). In addition to genome-wide hypomethylation, gene-specific hypermethylation events are also broadly observed in cancer (Esteller 2008; Ehrlich 2009). Aberrant hypermethylation generally occurs at CpG islands of promoters that are usually hypomethylated in somatic cells. Many CpG islands hypermethylated in cancer are in the promoters of genes involved in DNA repair, cell cycle regulation, and signaling pathway regulation.

Normal secretory luminal and contractile myoepithelial cells from mammary gland, the two principal lineages that are derived from a common precursor, occupy luminal cell–cell contact versus basal basement-membrane-rich microenvironments. Not surprisingly, cells enriched with lineage specific antibodies and magnetic beads

revealed lineage-specific gene expression patterns in addition to distinctive patterns of global methylation (Allinen et al. 2004). It is well known that altering cellular microenvironments will alter gene expression patterns, causing some genes to be expressed and others to be turned off. Because persistent transcriptional repression of a given CpG-containing promoter can favor increased methylation, whereas persistently active promoters favor unmethylated states (Turker 2002; Oyer et al. 2009), there is good reason to suspect that distinct microenvironments beget distinctive patterns of methylation. Serial analysis of gene expression in myoepithelial, endothelial, leukocyte, and stromal cells of normal breast tissue, ductal carcinoma in situ (DCIS), and invasive breast cancer samples further supported the view that the microenvironment is vastly different between normal and breast cancer tissue (Allinen et al. 2004). In this series of experiments, the most dramatic changes in gene expression were found in the myoepithelial cells and their matched stromal cells, and most of those changes corresponded to genes encoding secreted and cell surface proteins such as CXCL14, CXCL12, IGFBP7, and PDGFRL. Those results suggested a strong microenvironmental shift in DCIS and breast cancers. In a follow-up study, the researchers showed that there are alterations in DNA methylation of stromal and myoepithelial cells of DCIS and invasive breast tumors, compared with normal breast tissue, suggesting that the cells played a role in the generation of the abnormal tumor microenvironment and potentially contribute to tumor progression (Hu et al. 2005). The ECM also can exert an effect on target cells, leading to epigenetic changes in adjacent tissues. Preosteoblastic murine cells grown on collagen exhibited dramatic, global changes in DNA methylation patterns. Interestingly, the cells methylated the *Fas* promoter; a gene encoding a proapoptotic factor that induces programmed cell death in cells improperly attached to the ECM (Thaler et al. 2010).

Hypoxia, a key feature of the solid tumor microenvironment, can induce several types of epigenetic change in tumor cells. In studies using colon cancer and lung cancer cell lines exposed to hypoxic conditions, global hypomethylation was observed (Shahrzad et al. 2007). Additional studies have demonstrated that genes often methylated in breast carcinomas, such as *ERBB2* (formerly *HER2/neu*), are also methylated in associated stroma (Fiegl et al. 2006). Similarly, genes methylated in prostate carcinomas were also methylated in the adjacent stroma (Hanson et al. 2006). These examples suggest that there is a conversation between tumors, the ECM, and the stroma, perhaps mediated by secreted factors, that results in metastable epigenetic states that are characteristic of tumors. Overall, the tumor microenvironment, much like the normal tissue microenvironment, seems equipped to exert control of gene expression at the level of DNA methylation.

17.3.2 Chromatin Remodeling and Histone Modification

There is a crucial role of chromatin in the proper execution of developmental programs and maintenance of these programs in differentiated tissues. The basic unit of chromatin is the nucleosome, where approximately 150 base pairs of DNA

are wrapped around a histone octamer consisting of two copies each of histones H2A, H2B, H3, and H4. In eukaryotes, DNA packaged into chromatin ultimately achieves several-thousand-fold compaction. Although this organization provides ordering of DNA within the nucleus, it also generates a barrier to gene expression. To overcome this challenge, the cell is equipped with an arsenal of chromatin-modulating proteins, which serve as yet another means to regulate gene expression. Two major mechanisms of chromatin structure regulation have been identified. One consists of proteins that covalently change the chromatin structure by the addition of chemical moieties to the N-terminal tails of histones. The other mechanism consists of proteins that use the energy from ATP hydrolysis to mobilize nucleosomes and remodel chromatin.

Much like the role of "master regulator" transcription factors, proteins that drive lineage commitment and initiate patterns of gene expression, chromatin-modulating complexes can serve as master regulators of developmental programs by controlling the expression of tissue- and lineage-specific genes that are proximal to each other in three-dimensional space, but are not necessarily on the same chromosome (reviewed in Cremer and Cremer 2001; Joffe et al. 2010). For example, a fertilized egg must exponentially proliferate and give rise to cells that progressively differentiate into defined cell lineages and tissues. At the onset of this process, a global clearing of chromatin modifications occurs, including erasing DNA methylation and histone exchange. These marks are reestablished in the embryo as development progresses (Shi and Wu 2009). The importance of chromatin remodeling and histone-modifying factors in stem cell maintenance and self-renewal is well documented (Lessard and Crabtree 2010).

Studies examining the role of ATP-dependent chromatin remodelers in the tumor context are lacking. However, *Baf250a*, a component of the Swi/Snf complex, has been shown to be a critical regulator of stem cell pool size in the hematopoietic compartment (Krosl et al. 2010). Genetically modified mice harboring homozygous mutant alleles of *Baf250a* have a hematopoietic stromal component that drives the fetal liver microenvironment to produce twice as many hematopoietic stem cells as their littermate controls at embryonic day 14.5. Additionally, it has been demonstrated in mammary epithelial cells that ECM molecules and prolactin-directed gene expression requires the Swi/Sbt ATPase BRG1 as well as proper histone acetylation (Xu et al. 2007). As many factors that are critical in developmental process are often deregulated in cancer, and as many Swi/Snf components are mutated in cancer (Hargreaves and Crabtree 2011; Wilson and Roberts 2011), it is likely that components of the complex will also be identified as determinants of tumor-microenvironment-imposed gene expression.

Remarkably little is known about whether or not histone modifications can result from exposure to the tumor microenvironment, but a few examples hint at the likelihood of such an interaction. Histone modifications in tissues can also be perturbed by signals from the ECM. For example, in the presence of laminin-rich ECM, mammary epithelial cells undergo global histone deacetylation and distinct morphological change in shape. This decrease in histone H3 and histone H4 acetylation is followed by an increase in chromatin condensation and a decrease in gene

expression (Le Beyec et al. 2007). Hypoxia-inducible factors (HIFs) are transcription factors that mediate cellular adaptation to reduced oxygen environments. In a screen for HIF-regulated genes, several Jumonji C-domain-containing histone demethylase promoters were identified as direct binding targets of HIF-1α and HIF-2α (Pollard et al. 2008). Histone demethylases are a large family of enzymes that remove methyl groups from histone tails and, through this action, have the ability to influence transcriptional activation. Additional work has demonstrated that the genes encoding the Jumonji domain histone demethylases (*JMJD1A*, *JMJD2B*, and *JARIDA*) are hypoxia-inducible in an HIF-dependent manner (Krieg et al. 2010). Additionally, *JMJD1A* was found to be crucial to the proper hypoxic response as small interfering RNA depletion of transcripts resulted in an incomplete activation of HIF targets, owing to maintenance of silencing histone methyl marks on the promoter of these targets. As described above, histone demethylases seem to play an integral role in CSC maintenance and proliferation, and there are tantalizing links from the microenvironment to the regulation of these enzymes that may help explain the EMT and CSC states.

17.3.3 MicroRNAs

MicroRNAs (miRNAs) are a class of 21–25 nucleotide small noncoding RNAs that regulate gene expression at the posttranscriptional level. They were discovered through the analysis of developmental timing mutants in *C.elegans* (the heterochronic genes) (Lee et al. 1993; Wightman et al. 1993). Later, miRNAs were identified in both flies and humans. Currently, there are approximately 300 conserved miRNA genes, and deep sequencing has identified an additional 1,000 loci that produce RNAs resembling miRNAs (Mendell and Olson 2012). Instead of being translated into protein, mature miRNAs bind to messenger RNA and interfere with translation. Unlike DNA methylation, chromatin remodeling, or histone modification, miRNAs do not play an essential role in mammalian development, but rather seem to impact the response of fully differentiated tissues to stress stimuli. Most miRNAs are encoded by RNA polymerase II genes, which are usually spliced. Approximately one third of known miRNAs are embedded in introns of protein-coding transcripts and, in some cases, the miRNAs modulate the same biological process encoded by that gene (Winter et al. 2009). Unlike other small noncoding RNAs, such as piwiRNAs and small interfering RNAs, miRNAs fold back on each other to make a distinctive hairpin structure (Bartel 2004). This 60–80 nucleotide hairpin is processed by the sequential activity of Drosha and Dicer, RNAse type III endonucleases, to generate mature miRNAs 20–22 nucleotides in length (Winter et al. 2009).

An initial link between miRNAs and cancer was made in 2002 with the discovery that the miR-15a/miR-16-1 cluster is frequently deleted in chronic lymphocytic leukemia (Calin et al. 2002). It has become increasingly clear that all tumors examined to date exhibit dysregulated miRNA expression patterns, and each tumor

shows distinct expression patterns (Calin and Croce 2006). Additional work has demonstrated that miRNA profiles can be used to differentiate cancer type and stage, and are a better predictor of cancer stage and type than transcriptome profiles (Lee and Dutta 2009).

Although progress has been made in understanding the regulation of coding transcripts by miRNAs, little work has been done to uncover the regulation of these small noncoding RNAs themselves. Expression of miRNAs can be linked to functions associated with EMT and CSCs. Hypoxia induces specific families of miRNAs, including some of those involved in antiapoptotic response in hypoxic conditions as well as those overexpressed in many human tumors (Kulshreshtha et al. 2007). Recent work has highlighted an interesting interplay of epigenetic phenomena. With use of an in vitro model of endometrial cancer, it was demonstrated that downregulation of *miR-31* in cancer-associated fibroblasts led to an upregulation of the genome organizer and chromatin remodeling component SATB2. In turn, this upregulation of SATB2 led to greater migration and invasion efficiency of the adjacent endometrial tumor cells (Aprelikova et al. 2010). In work using noninvasive and invasive cell lines, it was shown that when invasive cells were grown in conditioned medium containing miR-17/miR-20, the ability of the invasive cells to migrate and invade across wounds or in three-dimensional collagen gels was significantly inhibited (Yu et al. 2010). The miRNAs miR-15 and miR-16 are downregulated in prostate tumors, leading to an increase in aberrant WNT3A and cyclin D1 activity, promoting proliferation, survival, and invasion (Bonci et al. 2008). A follow-up experiment demonstrated that prostate-cancer-associated fibroblasts also exhibit downregulation of miR-15 and miR-16, which resulted in promotion of tumor growth. This reduction of the levels of miR-15 and miR-16 in cancer-associated fibroblasts works through fibroblast growth factor 2 and fibroblast growth factor receptor 1 to support tumorigenesis (Musumeci et al. 2011). A reasonable explanation of this phenomenon is that yet-to-be proven tumor microenvironment factors, possibly fibroblast growth factors or even hypoxia, cause changes in regulation of miR-15 and miR-16 in both the stroma and the tumor.

17.4 Conclusions

The factors that regulate tumor initiation, growth, and survival are complex. Over the last several decades the role of the microenvironment and epigenetics have been shown to be crucial factors in oncogenesis. As we increase our understanding of the interplay between the microenvironment and epigenetics in both the normal context and the neoplastic context, our interrogation can be more sophisticated. To date the few studies linking the two have focused on the effects of microenvironmental factors on the epigenetic status of the tissue of interest. Future experiments should also focus on understanding the epigenetic changes in the microenvironment in response to signaling from tissues or tumors as well as how changes in epigenetic features such as DNA methylation and histone modification

can influence these signals. To do so will require more robust co-culture models as well as use of en vivo models. Better understanding of the cross talk between microenvironmental components and epigenetic regulation of gene expression will be key to make progress in the fields of both stem cell biology and cancer biology.

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Chapter 18
Tumor-Associated Neutrophils

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Abstract Neutrophils constitute an important component of the innate immune response and are classically involved in the defense against microorganisms due to their ability to produce several antimicrobial mediators. However, neutrophils can also be found in different sterile inflammatory responses foci, including in tumor adjacencies. Recent studies provided evidences that tumor-associated neutrophils (TAN) constitute a distinct population of neutrophils that can be polarized into an antitumoral phenotype (N1) or into a protumoral phenotype (N2). The fate and action of TAN will lastly depend on the factors present within the tumor microenvironment and will have profound implications in tumor biology, including tumor cell growth and survival, as well as invasiveness. In this chapter, we review the basic aspects of neutrophils and describe their function in tumor development in the light of their different phenotypes. Finally we address the manipulation of N1 and N2 phenotypes as a putative alternative approach for tumor therapy.

Keywords Tumor-associated neutrophils • Innate immune response • Neutrophils • Tumor biology • Antitumoral phenotype • Protumoral phenotype

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Abbreviations

Fas-L	Fas ligand
FPR	Formyl peptide receptor
G-CSF	Granulocyte colony stimulating factor
ICAM	Intercellular adhesion molecule
IFN	Interferon
Mac-1	Macrophage 1 antigen
MMP	Matrix metalloproteinase
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NK	Natural killer
ROS	Reactive oxygen species
TGF-β	Transforming growth factor β
TLR	Toll-like receptor
VEGF	Vascular endothelial growth factor

18.1 Neutrophils in Innate Immune Response: General Aspects

Neutrophils (or polymorphonuclear neutrophils) are leukocytes profoundly involved in the defense against microorganisms. Since their characterization by Ilya Ilyich Metchnikoff in 1893, these cells have been extensively associated with the tissue damage observed during acute infections. However, their real relevance in the regulation of the immune response has been revealed in the last few decades (Borregaard 2010; Mantovani et al. 2011). Neutrophils have a nucleus with a lobed appearance, where the separate lobes are connected by chromatin. Their Golgi apparatus is small compared with that of other cells of the same lineage, mitochondria and ribosomes are sparse, and the rough endoplasmic reticulum is not observed. They are the most abundant leukocytes in humans and they account for approximately 50–70 % of all white blood cells (leukocytes). The reference range of neutrophil counts in human blood samples is 2.5×10^9–7×10^9/L. They are generated in large numbers in the bone marrow and circulate in the bloodstream for a few hours (6–8 h in humans and 10–11 h in mice) (Summers et al. 2010). Most of their lifespan is spent in the bone marrow, and under physiological conditions less than 2 % of neutrophils are found in the bloodstream (Mary 1984, 1985; Semerad et al. 2002). Approximately two thirds of the bone-marrow hematopoietic activity is related to the generation of monocytes and granulocytes. The control of neutrophil homeostasis in the blood is regulated mainly by the proliferation/differentiation of precursors in the bone marrow, egress of mature neutrophils into the periphery bloodstream, and neutrophil clearance in the spleen, liver, and bone marrow (Semerad et al. 2002). Granulocyte colony stimulating factor (G-CSF) is essential for controlling the production of neutrophils during

infections, but G-CSF appears to be not necessarily required for granulocytopoiesis since G-CSF null mice have approximately 25 % residual granulocytopoiesis and are still able to produce mature neutrophils (Lieschke et al. 1994).

Among different adhesion molecules and chemokines involved in neutrophil development, the chemokine receptors CXCR4 and CXCR2 are essential for the homing of stem cells and mature neutrophils to the bone marrow (Lapidot and Kollet 2002; Eash et al. 2009, 2010). Whereas CXCR4 retains neutrophils in the bone marrow, CXCR2 facilitates their egress. CXCR4 ligand stromal-cell-derived factor 1 (CXCL12) and the CXCR2 ligands keratinocyte chemokine (CXCL1) and macrophage inflammatory protein 2 (CXCL2) are constitutively expressed by endothelial cells and osteoblasts in the bone marrow. The release of neutrophils into the periphery may be effectuated by signals through CXCR2, G-CSF receptor, or Toll-like receptors (TLRs) (Theilgaard-Monch et al. 2006). All of them form part of the mature neutrophil repertoire. Interestingly, the stimulation of these receptors does not cause additional neutrophil release from the bone marrow in the absence of CXCR4, confirming the fundamental role of CXCR4 signaling for homing of both immature and mature neutrophils in the bone marrow (Eash et al. 2009).

18.1.1 Neutrophil Granules

The presence of granules is a hallmark of granulocytes (eosinophils, basophils, and neutrophils). The appearance of granules represents the developmental transition from myeloblasts to promyelocytes and the formation of granules continues until the segmented stage of maturation is reached. These granules may be filled passively by a flow of proteins coming from the more proximal Golgi compartments (Borregaard and Cowland 1997; Borregaard 2010). The granules observed in mature neutrophils contain a variety of proteins involved in the antimicrobial host defense (Borregaard and Cowland 1997). The populations of granules in mature neutrophils are classified by histochemical staining as peroxidase-positive and peroxidase-negative: primary (azurophil) granules (i.e., myeloperoxidase), secondary (specific) granules (i.e., lactoferrin), and tertiary (gelatinase) granules (Table 18.1). They are functionally distinguished by the contents of their matrices as well as by their integral membrane protein. The latter point is most notable for the peroxidase-negative granules, which include specific and gelatinase-containing granules. The enveloping membranes contain a variety of functionally important membrane proteins, including β_2 integrins, the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase component flavocytochrome *b*-558, and the formyl peptide receptor (FPR) (Borregaard and Cowland 1997; Nauseef 2007). During neutrophil stimulation, granules fuse with the immature phagosome and release their contents into the phagosome, thereby exposing the ingested microbe to a large variety of toxic substances with relatively redundant activities. Stimulated polymorphonuclear neutrophils exhibit a notable increase in

Table 18.1 Contents of human neutrophil granules and secretory vesicles

Azurophil granules		Specific granules		Gelatinase granules		Secretory vesicles	
Membrane	Matrix	Membrane	Matrix	Membrane	Matrix	Membrane	Matrix
CD63	Acid β-glycerophosphatase	CD11b/CD18	Cathelicidin (hCAP-18)	CD11b/CD18	Acetyltransferase	Alkaline phosphatase	Tetranectin
CD68	Acid mucopolysaccharide	CD15	Collagenase	Cytochrome-b_{558}	Annexin I	CD10	Hepatocyte growth factor
Presenilin-1	α_1-Antitrypsin	CD66a	Gelatinase	Diacylglycerol deacetylating enzyme	Gelatinase	CD11b/CD18	Plasma proteins (albumin)
uPA receptor	Azurocidin/CAP37/heparin-binding protein	CD66b	Heparanase	fMLP receptor	Heparanase	CD13	
V-type H^+-ATPase	Bactericidal/permeability-increasing protein	Cytochrome-b_{558}	Hepatocyte growth factor	Leukolysin	Hepatocyte growth factor	CD14	
	Cathepsins	fMLP receptor	Histaminase	NRAMP-1	Lysozyme	CD16 (FcRIII)	
	Defensins	Fibronectin receptor	Human neutrophil lipocalin	SCAMP	β_2-Microglobulin	CD45	
	Elastase	G protein α subunit	Lactoferrin	SNAP-23, SNAP-25	SGP28 (CRISP-3)	CR1 (CD35)	
	β-Glucuronidase	Laminin receptor	Lysozyme	uPA receptor	uPA	C1q receptor	
	β-Glycerophosphatase	Leukolysin	β_2-Microglobulin	VAMP-2		Cytochrome *b*-558	
	Lysozyme	NB1 antigen	NGAL	V-type H^+-ATPase		Decay-accelerating factor	
	α-Mannosidase	19-kDa (P1C3) protein	SGP28 (CRISP-3)	1-Diacylglycerol lipase		fMLP receptor	
	Myeloperoxidase	Rap1, Rap2	Sialidase	Ubiquinone		Leukolysin	
	N-Acetyl-β-glucosaminidase	SCAMP	uPA			VAMP-2	
	Proteinase-3	SNAP-23, SNAP-25	Vitamin B_{12}-binding protein			Phospholipase D	
	Sialidase	Stomatin	YKL-40 (human cartilage glycoprotein 39)				
	Ubiquitin–protein conjugates	Thrombospondin receptor					
	uPA	TNF receptor					
		uPA receptor					
		VAMP-2					
		Vitronectin receptor					
		IL10 receptor					

fMLP N-formyl-methionine-leucine-phenylalanine, *uPA* urokinase-type plasminogen activator

oxygen consumption, which is called "phagocyte respiratory burst" (Babior et al. 2002). The enzyme responsible for the respiratory burst is a complex of membrane and cytosolic proteins with constituents segregated into distinct compartments in the resting neutrophil (Borregaard and Tauber 1984). Resting neutrophils contain three proteins essential for NADPH oxidase activity: p47phox, p67phox, and rac2 (Volpp et al. 1988; Kwong et al. 1993). Deficiency in these proteins may occur and result in chronic granulomatous disease (Clark et al. 1989; Ambruso et al. 2000). These specific oxidase complexes transfer electrons from the NADPH present in the cytosol, sequentially through two nonequivalent hemes in flavocytochrome *b*-558 (Cross et al. 1995), and across the membrane to the electron acceptor, molecular oxygen, which results in the generation of the superoxide anion. Superoxide anion produced in the phagosome is able to interact with itself, thereby dismutating to produce H_2O_2, with other targets in the phagosome. Both phenomena appear to operate together in the mature activated neutrophil.

During the processes of phagocytosis and microbial killing, the microenvironment in the phagosome presents a high level of toxicity. The very dynamic nature of phagosome constituents and their multiple associations is a challenge in attempts to understand their antimicrobial action. There is an important degree of promiscuity in the interactions of the released products with microbial targets, other granule proteins, and host components in the phagosome. NADPH oxidase activation creates a flux of oxidants, which in turn react with each other as well as with many granule contents to generate a series of chemical derivatives, each with its own specific activity (Weiss et al. 1983; Pattison and Davies 2006).

The structural heterogeneity of neutrophil granules is a natural consequence of differences in the expression of granule proteins during the maturation of myeloid cells in the bone marrow. However, it is less apparent how the sequential steps for mobilization of neutrophil granules are established. Secretory vesicles are mobilized completely by different stimuli that are important in the interaction of neutrophils with the activated endothelium, such as signaling by selectins and chemotactic factors, for example, IL-8 (human analogue of mouse CXCL1) and *N*-formyl-methionine-leucine-phenylalanine. In this way, secretory vesicles can provide the surface membrane with receptors and different functional proteins (i.e., integrins) for use in transmigration without releasing potentially toxic granule proteins (Borregaard et al. 1992; Sengelov et al. 1995).

18.1.2 Neutrophil Activation

Circulating neutrophils can be rapidly recruited from the peripheral blood into peripheral tissues during infection or sterile tissue injury. This interference in tissue homeostasis can be molecularly recognized either by professional tissue-resident cells, such as macrophages and mast cells, or by stromal cells (Arancibia et al. 2007; Zeytun et al. 2010). A plethora of different stimuli, especially pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns, activate sentinel cells to

release proinflammatory mediators (i.e., IL-1β, TNF-α, chemokines, and lipid mediators such as platelet-activating factor (Zeytun et al. 2010; Williams et al. 2011). It is well established that *N*-formyl peptides are able to induce neutrophil chemotaxis and activation trough the seven-transmembrane G-protein-coupled receptor FPR1. The production of formylated proteins is restricted to bacteria and mitochondria, and therefore FPR1 might be characterized as a PAMP, directly recognizing molecules originating from microbes and apoptotic/necrotic cells (Zhang et al. 2010). In different experimental systems, mitochondria-derived formyl peptides, when injected, can induce neutrophil recruitment and trigger inflammation. In conjunction with intravascular CXCL2 released concomitantly with the antimicrobial response, formyl peptides guide neutrophils into sites of sterile inflammation (McDonald et al. 2010; Zhang et al. 2010). Neutrophils express different classes of PAMP receptors, including all members of the TLR family (except TLR3). Moreover, they express C-type lectin receptor dectin 1 (also known as CLEC7A), C-type lectin-like receptor 2 (also known as CLEC7A), C-type lectin-like receptor 1 (also known as CLEC1B), and cytoplasmic sensors of ribonucleic acids (RIG-I and melanoma-differentiation-associated protein 5) (Hayashi et al. 2003; Tamassia et al. 2008; Kerrigan et al. 2009). Importantly, the sensing of pathogens and tissue damage through these PAMPs, together with other signals (i.e., from resident cells), fully activates the effector functions of neutrophils (Nathan 2006; Borregaard 2010; Mantovani et al. 2011). These include the production of reactive oxygen species (ROS), lytic enzymes, antimicrobial peptides, and different cytokines.

The production of cytokines by neutrophils is tightly controlled by mechanisms that act at different cellular levels, including messenger RNA transcription, stability, or translation, but also protein secretion (Cassatella 1999). Regarding protein secretion, important molecules such as TNF-related apoptosis-inducing ligand , CXCL8 (IL-8), CCL20, and IL-1 receptor antagonist are not released following synthesis but are stored intracellularly prior to release on stimulation (Scapini et al. 2008). Neutrophil-derived cytokines may have important functions other that those related to acute inflammatory responses to pathogens. Human and murine neutrophils have been shown to upregulate the expression of membrane-bound RANKL (the ligand for receptor activator of NF-κB) following activation in vitro and in vivo, which results in an important participation in the process of bone reabsorption (Chakravarti et al. 2009). In addition, neutrophils from synovial fluid of patients with rheumatoid arthritis, or in different types of B cell malignancies and solid tumors, express and secrete high levels of APRIL (a B-cell-proliferation-inducing ligand), which promotes survival and proliferation of normal and malignant B cells (Gabay et al. 2009; Roosnek et al. 2009).

18.1.3 Recruitment of Leukocytes to Inflamed Sites

As discussed already, neutrophils from peripheral blood are capable of sensing danger signals and migrating toward damaged tissues to perform bacterial killing or amplify the local inflammatory response (Nathan 2006; Heissig et al. 2010).

Endothelial barrier dysfunction, an active event that results from the opening of cell–cell/matrix junctions and/or increased vesicle-mediated transcytosis, facilitates the movement of neutrophils across the endothelium. Importantly, vasoactive substances and cytotoxic agents (such as ROS and proteases) released by activated neutrophils may increase or prolong endothelial permeability during inflammation (Boueiz and Hassoun 2009; Pun et al. 2009).

One of the pivotal steps during neutrophil recruitment to injured tissues is the activation of adhesion molecules on the endothelium neighboring inflamed sites. It has been suggested that neutrophils approach the inflammatory site as closely as possible within the blood vessels. In this case, the blood vessels will function as fast-track transfer routes for neutrophils (McDonald et al. 2010). After this first moment of approach to the injured site, neutrophils will eventually leave the peripheral blood and transmigrate into peripheral tissues, depending on different positive signals from proinflammatory molecules. This migration event mainly occurs at the postcapillary venules (Sadik et al. 2011; Williams et al. 2011). The leukocyte adhesion cascade is a sequence of active physical interactions between neutrophils and endothelial cells (Williams et al. 2011). It was originally thought to consist of three distinct steps, notably rolling, adhesion, and transmigration (diapedesis). Studies in the last decade have shown that more than three steps can be distinguished. Different molecular mechanisms involved in slow rolling, adhesion strengthening, and intraluminal crawling are regarded as additional new steps in the already complex neutrophil transmigration process (Ley et al. 2007; Borregaard 2010).

The attachment of neutrophils to endothelial cells is directly determined by the endothelial cells themselves, which respond to stimuli such as TNF-α and IL-1β generated during the inflammatory response. This stimulation results in expression of P-selectin, E-selectin, and members of the integrins [intercellular adhesion molecules (ICAMs) and vascular cell adhesion molecules) on their luminal surface. These selectins bind P-selectin ligand 1 and L-selectin, both expressed constitutively on the neutrophil surface (Bruehl et al. 1997; Buscher et al. 2010). The initial contact between neutrophils and activated endothelial cells is established by the binding of P-selectin ligand 1 to P-selectin and E-selectin. There is a sequential binding and detachment process between selectin-mediated bonds, but it is sufficiently strong to mediate attachment during the shear stress that is created by the laminar flow of blood in inflamed vessels (Ley et al. 2007; Pospieszalska et al. 2009). Among the molecules that mediate the firm adhesion and rolling of neutrophils in the activated endothelium are the β_2 integrins lymphocyte-function-associated antigen 1 ($\alpha_L\beta_2$) and macrophage 1 antigen (Mac-1; $\alpha_M\beta_2$), which are present on neutrophils, and their ligands, members of the immunoglobulin superfamily, ICAM-1 and ICAM-2, which are present on the endothelial cells. During all these multistep interactions in endothelial cells, neutrophils are activated by chemokines, which are bound to the endothelium via glycosaminoglycans (Johnson et al. 2005). Activation of neutrophils by chemokines enhances the binding affinity of β integrins and subsequently induces a more effective arrest. Once activated and arrested, the neutrophils start the transmigration process. They

are able to transmigrate through junctions between endothelial cells (paracellular) or through an endothelial cell (transcellular) (Lou et al. 2007). The transcellular route is taken by approximately 20 % of neutrophils, but the proportion may differ among different tissues and depends on the stimulation status of the endothelial cells (Woodfin et al. 2010). It has been shown that the lateral migration of neutrophils mediated by Mac-1 shifts the neutrophils to paracellular migration, and the proportion of transcellular migration increases from 20 to 80 % in the absence of Mac-1 as determined in vivo (Phillipson et al. 2008). After crossing the endothelial lining, neutrophils make their way into the basal membrane. There is an extensive release of proteases, which are capable of cleaving membrane collagens, and laminins such as elastase (azurophil granules), matrix metalloproteinases (MMPs; MMP8 and MMP9), and the membrane-attached MMP MT6-MMP (Kang et al. 2001). The detailed mechanism of penetration of the basal membrane is still elusive. Emigrated neutrophils are more active as phagocytic cells than circulating neutrophils (Sorensen et al. 2001) and activate a proinflammatory program that results in the generation of chemokines, including IL-8 (CXCL8) and growth-related oncogene α (Scapini et al. 2005; Theilgaard-Monch et al. 2006), which will recruit additional inflammatory cells and amplify the response. Lipid mediators such as leukotriene B_4 are rapidly produced and have a short half-life, mainly acting at the beginning of neutrophil recruitment cascades and at the site of injury and inflammation. On the other hand, chemokines are able to act later in recruitment events and, more importantly, at longer distances. The pattern of production of chemokines is slower than that of lipid chemoattractants. Moreover, chemokines are regulated at transcriptional levels and their release may be controlled by posttranscriptional regulatory mechanisms (Young et al. 2007; Borregaard 2010).

18.1.4 Interactions of Neutrophils with Other Immune Cells

Cytokines and growth factors present at sites of inflammation or infection can influence some phenotypical features of neutrophils within the tissues. If these signals reach the bone marrow, they may influence the phenotype of neutrophils during hematopoiesis. However, because of the short life span of these cells and their lack of proliferative potential, it is not yet clear to what magnitude locally induced phenotypic changes in neutrophils are stable or fully reversible. Signals such as adhesion, transmigration, hypoxia, binding of microbial products, and cytokines can delay their programmed cell death and thus extend their survival in vivo (Colotta et al. 1992; Mantovani et al. 2011). Macrophages attract neutrophils to the inflammatory site and produce cytokines to control the survival and activity of the recruited leukocytes. In addition, human mesenchymal stem cells activated through TLR3 or TLR4 signaling can also play a pivotal role and increase the life span and activation of neutrophils (Brandau et al. 2010; Soehnlein and Lindbom 2010; Cassatella et al. 2011). Neutrophils have also been shown to cooperate with

dendritic cells during infection, and supernatant from cultures of mouse neutrophils stimulated with *Toxoplasma gondii* induces the maturation of bone-marrow-derived dendritic cells in vitro as well as their production of IL-12 and TNF-α (Bennouna and Denkers 2005). In addition, human neutrophils induce the maturation of monocyte-derived dendritic cells through contact-dependent interactions in vitro (Megiovanni et al. 2006), and during their interaction, human neutrophils may potentiate the release of interferon (IFN)-γ by natural killer (NK) cells, without regulating their cytotoxicity (Costantini et al. 2011).

In terms of interaction between T cells and neutrophils, it is well known that these cells can modulate each other's recruitment to inflamed sites. Activated neutrophils can attract T_h1 and T_h17 by the release of CCL2, CXCL9, and CXCL10 or CCL2 and CCL20, respectively (Pelletier et al. 2010). In addition, activated T cell populations can recruit neutrophils, although the mechanism used by individual T cell subsets differs and the detailed mechanisms are lacking (Pelletier et al. 2010; Mantovani et al. 2011). Both IL-17A and IL-17F released by T_h17 cells can stimulate epithelial cells to secrete factors involved in granulopoiesis (G-CSF and stem cell factor), as well as neutrophil chemotactic factors (such as CXCL1, CXCL2, and CXCL8) (Pelletier et al. 2010; Abi Abdallah et al. 2011). Neutrophils can interfere with the ability of mature activated dendritic cells and macrophages to present antigens after their migration into the lymph node, mainly by competing with the antigen-presenting cells for the available antigen in a major histocompatibility complex class II dependent manner (Abi Abdallah et al. 2011). In summary, neutrophils can influence the maturation of dendritic cells and then contribute to the proliferation and polarization of lymphocytic populations. It is becoming clear that neutrophils appear to exert a regulatory role in vivo at both peripheral sites and lymph nodes (Galli et al. 2011; Mantovani et al. 2011).

18.2 Tumor-Associated Neutrophil Polarization: N1 and N2

A mounting body of work has been devoted to the double-faceted role of neutrophils in tumor development. Despite very convincing data and elegant articles, inconsistencies preclude conclusive statements regarding whether neutrophils contribute to tumor killing or progression. The hypothesis that leukocytes are correlated to tumors derives from initial studies reporting the constant presence of inflammatory cells, including macrophages, neutrophils, and NK cells, surrounding and infiltrating the tumor mass (Balkwill and Mantovani 2001; Piccard et al. 2012). This may be an immune system strategy to confine and combat malignant cells, and several studies report that neutrophils efficiently kill cancer cells both in vitro and in vivo. A direct extrapolation of these facts is that tumor cells might release chemoattractants (or necrotic products per se) to guide the influx of leukocytes into the tumor mass. However, in a teleological approach, why is the behavior of attracting "killing-driven" neutrophils to the vicinity positively selected by some tumors? The answer to this question relies on the ability of the tumor

to silence these leukocytes and profit from their content to nourish tumor growth and spread. Similarly to macrophage polarization—the "classical M1" and the "tolerogenic M2" (Solinas et al. 2009)—the paradoxical neutrophil character was baptized as the "antitumoral N1" and "protumoral N2" (Fridlender et al. 2009), whereas a naïve, quiescent neutrophil is referred to as "N0" (Houghton 2010).

Neutrophils infiltrate within inflamed tissues following chemotactic clues that orchestrate several steps of leukocyte recruitment. Therefore, these cells are attracted to tumors because malignant cells directly produce chemokines and secrete proinflammatory cytokines, which will leads to production of chemokines by parenchymal cells. Almost all tumors release chemokines, including CCL3, CXCL8, CXCL6, CXCL1, and CXCL2, which attract and activate neutrophils (Hirose et al. 1995; Loukinova et al. 2000; Piccard et al. 2012). Alternatively, tumor cells secrete IL-1, IL-2, IL-4, IL-7, IL-10, IL-12, G-CSF, TNF-α, and IFN-α, which will induce secondary chemokine release by nontumor cells (Di Carlo et al. 2003). Depending on the cytokine environment found by arriving neutrophils, an antitumoral or a protumoral phenotype will be acquired, which has been highlighted as an important feature of tumor fate.

18.2.1 The Antitumoral Neutrophil Phenotype: N1

The contribution of neutrophils to immunity against invading microbes is now undisputable. These cells avidly recognize and eliminate pathogens using several strategies, including microbial phagocytosis, direct membrane damage by granule enzymes, and by releasing DNA-protein extracellular traps (neutrophil extracellular traps) (Amulic et al. 2012). Neutrophils are also able to track and follow pathogen-derived molecules (including DNA and formyl peptides) released within the tissue, but the battle may also occur inside blood vessels (Hickey and Kubes 2009; McDonald et al. 2010). When microbes spread using the vasculature, a systemic inflammatory response is triggered and the host succumbs. In this context, the intravascular immunity is key to avoiding pathogen spread with consequent septic shock. More recently, neutrophils have also been increasingly linked to the recognition of sterile cell death (specially necrotic tissues) (McDonald et al. 2010), where they may contribute not only to tissue repair, but also to injury amplification (Imaeda et al. 2009; Marques et al. 2012). Recognition of necrosis-derived products will be relevant in the context of neutrophil accumulation in larger tumor masses, and will be discussed later.

Neutrophils also directly kill tumor cells, and the earliest reports of this ability date from the 1970s (Fridlender and Albelda 2012). Although the precise neutrophil tumoricidal strategies are still not completely understood, bona fide neutrophil cytotoxic agents, such as defensins, ROS, and proteases, are toxic to tumor cells. If we take into account that almost all of these are "short-range weapons," neutrophils may require intimate contact with target cells in order to generate an efficient cytotoxic microenvironment. Such an antitumoral phenotype

is achieved when neutrophils reach tumor cells displaying an activated, proinflammatory behavior. Morphologically, these cells exhibit the regular lobulated and segmented nuclei (in contrast to the round N2 nuclei; described later) (Fridlender et al. 2009). This "classical mode" is accepted as a default fate, and in this case, tumor strategies to dampen neutrophil activation have failed. Neutrophils may acquire (or keep) an N1 profile when they are exposed to lower levels of transforming growth factor β (TGF-β) and IL-10 or increased levels of proinflammatory cytokines, including IL-1β and TNF-α. Briefly, they are identified as $CD11b^{+}LyG6^{+}$ cells (LyG6 is lymphocyte antigen 6 complex, locus G) with elevated levels of CCL3 and ICAM-1, high superoxide production ability, and lower levels of arginase, vascular endothelial growth factor (VEGF), CCL, CXCR4, and gelatinases (Fridlender et al. 2009; Houghton 2010; Fridlender and Albelda 2012; Piccard et al. 2012). Considering that arginine is a factor needed for proper tumor-killing activity of cytotoxic T cells ($CD25^{+}CD137^{+}$), lower levels of arginase 1 in N1 neutrophils will boost T cell activation and proliferation (Rotondo et al. 2009). Data regarding the role of Fas/Fas ligand (Fas-L) in the neutrophil–tumor relationship are still being debated. Briefly, Fas (also called CD95) is a cell surface receptor protein that belongs to the TNF receptor family. When Fas is combined with Fas-L, Fas-L triggers a death signal to Fas^{+} cells. Antitumoral neutrophils have reduced Fas-L expression; therefore, they may fail to induce direct tumor cell apoptosis via Fas. However, as most N1 cells are Fas^{+}, and tumor cells may induce neutrophil apoptosis via Fas-L, evading the immune response (Piccard et al. 2012).

18.2.2 The Protumoral Neutrophil Phenotype: N2

In sharp contrast to the regular cytotoxic behavior exerted by neutrophils, these cells can be educated to collaborate with tumor cells depending on in which cytokine microenvironment their encounter happens. Initial speculations that neutrophils might "score an own goal" date from the nineteenth century, when it was assumed that since they are present in all infection sites, neutrophils may be used as vehicles for spread of microbes (Amulic et al. 2012). Although these initial conclusions were considered wrong, recent reports confirmed that phagocytized parasites, including *Leishmania major*, might remain viable and use neutrophils to evade the immune system and disseminate infection (Peters et al. 2008). In the last case, the absence of neutrophils reduced, rather than enhanced, the ability of parasites to succeed in infection. Since neutrophils are also frequently associated with tumor infiltrate, the same rationale guided the hypothesis that they could also be linked to tumor progression. Numerous conflicting data have been published in this field since the late 1980s and early 1990s, and the controversies probably arose from the different models and tumor lineages used in the studies. In 2009, Fridlender et al. (2009) provided strong data supporting the existence of a neutrophil subpopulation that may have protumoral activity (called as N2).

An N2 subpopulation is $CD11b^{+}Ly6G^{+}Ly6C^{-/int}$, expressing elevated levels of CXCR4, CCL2, VEGF, and MMP9. In addition, these cells have reduced ICAM-1 and Fas expression, and produce less CCL3 and TNF-α (Fridlender et al. 2009; Houghton 2010; Piccard et al. 2012). Morphologically, N2 neutrophils have circular nuclei, in contrast to the segmented regular nucleus observed in the N1 subpopulation (Fridlender et al. 2009). It has become well accepted that the presence of TGF-β in the tumor environment is determinant to polarize emigrated neutrophils into an N2 phenotype. Also, in hypoxic regions and in the absence of proinflammatory cytokines (particularly IFN-β), increased amounts of CXCL-12, IL-6, and CCL2 will increase the life span of neutrophils within the tumor, enhancing their protumoral role (discussed later). In accordance with this, protumoral neutrophils express lower surface levels of Fas receptor, which may contribute to their extended viability in the tumor area.

18.3 Tumor-Associated Neutrophils in Tumor Biology

18.3.1 Tumor-Induced Neutrophilia: Suicidal Tendencies?

As already mentioned, the tumor microenvironment contains a plethora of factors that control the recruitment of neutrophils to the areas of tumor development and their activation. Such factors, produced by the tumor cells themselves and/or by the nearby stromal and inflammatory cells, may regulate neutrophils in a multifaceted process, including granulopoiesis, transmigration from the blood vessels to tissues, and neutrophil differentiation (Fig. 18.1). To illustrate this scenario, patients who develop various types of tumors frequently exhibit a remarkable increase in the circulating number of neutrophils that can be up to 50-fold the normal levels of these cells in the bloodstream (Welch et al. 1989). Although the reason for such drastic neutrophilia is not known, some putative factors such as granulocyte–macrophage colony stimulating factor, G-CSF, VEGF, IL-1β, and IL-6 have been related to this process and some of them were shown to induce bone-marrow-mediated granulopoiesis (McGary et al. 1995; Lechner et al. 2010).

Increased levels of neutrophils within the circulation do not necessarily mean a more efficient response against tumor cells. This may be explained in part by the inhibition of neutrophil activation or influx to tumor sites by factors produced, for example, by neutrophils themselves (discussed earlier) and/or by other inflammatory cells. Moreover, even abundant neutrophils adjacent to tumor cells are not a guarantee of tumor resolution. A recent concept in tumor biology is the ability of the tumor microenvironment to modulate neutrophil characteristics. In this sense, as soon as neutrophils pass the blood vessel walls, they are exposed to a very peculiar microenvironment that can result in a dichotomy. Depending on the composition of the tumoral milieu, neutrophils can assume either an antitumoral phenotype (N1) that will effectively fight against tumor development, or a protumoral

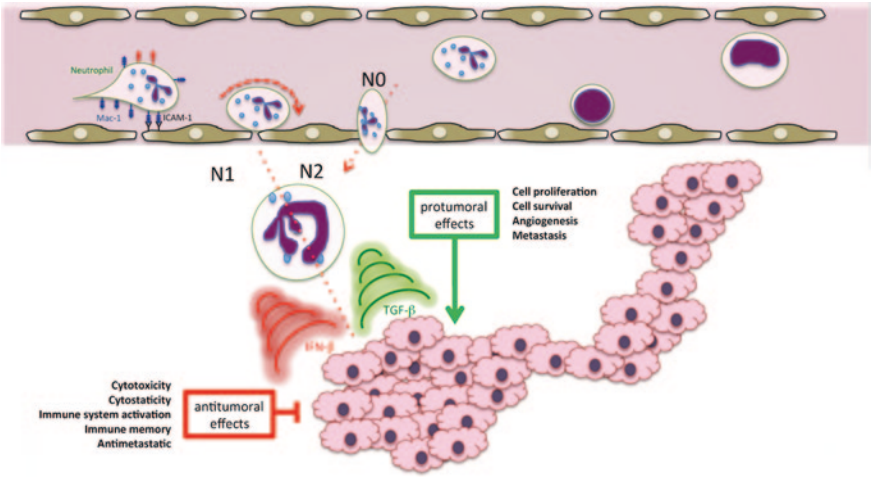

Fig. 18.1 The dual roles played by tumor-associated neutrophils in tumor biology. After recruitment to the area of tumor development, quiescent neutrophils (N0) can be polarized into N1 or N2 phenotypes depending on the factors present in the tumor microenvironment, and exert an antitumoral or a protumoral effect

phenotype (N2) that, in contrast, supports tumor development stages, including metastasis (Fig. 18.1) (Fridlender et al. 2009).

18.3.2 N1 Neutrophils and the Antitumoral Activity

When polarized into an N1 phenotype, neutrophils were shown to be cytotoxic and cytostatic against various tumor types both in vitro and in vivo. The direct cytotoxic effects of N1 neutrophils are mediated in part by the production of ROS, which culminates in the lysis of tumor cells (Lichtenstein et al. 1989; Zivkovic et al. 2005, 2007; Fridlender et al. 2009). Moreover, activated N1 cells produce and release other soluble mediators of cell killing, such as TNF-α, IL-1β, IFNs, and hypochlorous acid (HOCl), that in addition to a cytotoxic effect promote local microvessel injury and extracellular matrix degradation (Dallegri et al. 1991; Zivkovic et al. 2005; Borregaard 2010). On the other hand, N1 activated neutrophils may exert a cytostatic effect on tumor cells by the production of singlet oxygen ($^1O_2$) that will dampen tumor cell proliferation (Zivkovic et al. 2005). Taken together, both the cytotoxic activity and the cytostatic activity of neutrophils toward tumors may act synergistically in controlling the size of the tumor (stabilization or reduction) or even promoting its complete regression. This beneficial response will lastly depend on the activation status of neutrophils (Coussens and Werb 2002).

Although neutrophils are important constituents of the inflammatory cell infiltrate in the tumor microenvironment, they are not the only cell type present in vicinity of the tumor. Other innate immune cells such as macrophages, dendritic cells, and NK cells as well as components of the adaptive immune response such as B and T lymphocytes can also be observed in close association with tumor cells. Therefore, the interaction of neutrophils and other immune cells is of great interest and provides important information about how tumor development can be controlled. In this context, recruited neutrophils produce different soluble factors such as chemokines (CCL3, CXCL9, and CXCL10) and proinflammatory cytokines (e.g., IL-12, TNF-α, and granulocyte–macrophage colony stimulating factor) that are involved in the recruitment and activation of $CD8^+$ T lymphocytes. The $CD8^+$ T cells increase the cytotoxic response against tumor cells and, in some cases, mediate complete tumor rejection (Scapini et al. 2000; Fridlender et al. 2009). Nonetheless, neutrophils can communicate with dendritic cells through direct cell–cell contact or by the mediation of TNF-α, activating another important innate immune cell to fight tumor cells (Fridlender and Albelda 2012). There is also a growing body of evidence showing that neutrophils are able to activate $CD4^+$ T lymphocytes that will orchestrate other immunological events in response to tumor cells, including a long-lasting tumor-specific immune memory that will act against subsequent tumor initiation (Cavallo et al. 1992; Di Carlo et al. 2003).

Another striking antitumoral role of neutrophils concerns the early stages of tumor development. When activated N1 cells degranulate, one of the products released is MMP8. Deficient MMP8 expression was significantly related to skin chemical carcinogenesis, suggesting a protective role of this collagenase in tumor development. Although the mechanism for such an effect is not completely understood, it is likely that MMP8 somehow prevents the establishment of a chronic inflammatory response and the recruitment of neutrophils to the site of the carcinogenesis challenge (Fridlender and Albelda 2012; Piccard et al. 2012).

18.3.3 N2 Neutrophils and Tumor Support

There is considerable evidence that in some tumor models, neutrophils may assume a protumoral role rather than an antitumoral role. Of particular interest are studies that systematically addressed the prognosis of neutrophilia in patients with advanced stages of cancer. Many of these studies revealed that the severer the increase in the levels of circulating neutrophils, the poorer the prognosis of the patients was (Schmidt et al. 2007; Teramukai et al. 2009; Tavares-Murta et al. 2010; Dumitru et al. 2012). However, despite the evidence for a dual role of neutrophils in tumor biology, the real participation of these cells in tumor progression is still under debate. Recently, a brilliant study by Fridlender et al. (2009) enlightened this discussion. In their study, they showed that depending on the tumor microenvironment in which neutrophils are immersed, they could assume distinct phenotypes. For instance, in low TGF-β and high IFN-β concentrations,

neutrophils are polarized into the antitumoral N1 phenotype. On the other hand, high concentrations of TGF-β and low levels of IFN-β result in the polarization of neutrophils toward the protumoral N2 phenotype and inhibition of the N1 subtype.

Nowadays, the debate is whether N1 and N2 neutrophils are distinct cell populations that differentiate within the tumor microenvironment or whether they represent different activation states of the same population of cells. It was hypothesized that the N1 phenotype could be nothing but more greatly activated neutrophils, whereas the N2 phenotype would consist of moderately activated cells that would not produce as efficiently the characteristic factors released in the tumor microenvironment (Houghton 2010; Gregory and Houghton 2011). In contrast with this hypothesis, an analysis of the gene expression profile of N1 and N2 neutrophils revealed that although most of the differences between both phenotypes concerned the upregulation of the same genes in N1 cells, some genes were indeed upregulated in N2 cells, suggesting different transcriptional programs between both phenotypes (Fridlender et al. 2009, 2012). It is clear that studies dealing with the "manipulation" of N1 to N2 cells and vice versa are needed to overcome this question. Regardless of this discussion, N2 neutrophils were clearly shown to exhibit protumoral characteristics.

Once neutrophils are polarized into the N2 phenotype, they express and release proteases, cytokines, and chemokines that support tumor initiation and growth. N2 cells release MMP9, a protease that substantially modifies the extracellular matrix in the vicinity of the tumor. These modifications are directly related to tumor initiation as they promote the release of entrapped growth factors from the extracellular matrix as well as reduced apoptotic rates of tumor cells (Bekes et al. 2011; Gregory and Houghton 2011). Another important protease stored in neutrophil granules and released in response to activation is the neutrophil elastase. It is speculated that neutrophil elastase diffuses between the tumor mass, enters the cells by a clathrin-mediated endocytosis, and binds to insulin receptor substrate 1, resulting in its degradation. As a consequence, the Akt pathway is activated and signals for cell proliferation and survival (Houghton 2010; Houghton et al. 2010). In addition, as a consequence of polarization, N2 cells have a high profile of chemokine and cytokine activity. Protumoral neutrophils produce increased levels of CXCL2 (about 188-fold), CXCL1 (about 140-fold), CCL3 (about 76-fold), CXCL6, and CXCL8, which are potent attractants and activators for other neutrophils. Moreover, these cells play a pivotal role in the recruitment of other immune cell types that will be sculpted to support tumor growth through the secretion of other factors such as CCL8, CCL12, CCL17, CXCL9, and CXCL16 (Fridlender and Albelda 2012). Besides the regulation of other cell types, the neutrophil cytokines may also be related to tumor onset, as prolonged exposure to such factors is thought to trigger genomic instability (Aivaliotis et al. 2012).

Following tumor onset, an increase in the tumor mass is observed that corresponds to tumor cell proliferation. At this point, the energetic and oxygen supplies are one of the key issues for tumor survival. It is not rare to observe necrotic tumor cells within the core of tumor areas, especially because this is a region in which hypoxia may occur (Grivennikov et al. 2010; Piccard et al. 2012). The released

necrotic products, also known as damage-associated molecular patterns, have the capacity to recruit and accumulate neutrophils in areas of cell damage (Rock et al. 2010). What is surprising in tumor-induced angiogenesis is that hypoxic regions, to which neutrophils are attracted, drive the polarization of neutrophils to the N2 phenotype owing to low concentrations of IFN-β (Piccard et al. 2012). In this case, neutrophils are stimulated to increase the expression of VEGF, CXCR4, and MMP9, which are potent factors related to angiogenesis (Jablonska et al. 2010). Also, these cells release proangiogenic factors such as MMP9 and oncostatin M that control the bioavailability of angiogenic molecules (i.e., VEGF and fibroblast growth factor) and angiostatic factors retained within the extracellular matrix (Queen et al. 2005; Bekes et al. 2011; Gregory and Houghton 2011). As a result of angiogenesis, oxygen and nutrient supply to the growing tumor is restored.

Another intriguing role of N2 neutrophils is their relation to tumor invasiveness. Metastasis can be generally described as the growth of tumor cells beyond their original boundaries by the invasion of adjoining tissues or organs. This process is one of the most challenging situations in clinical practice, as over 90 % of cancer mortality is caused by metastasis (Grivennikov et al. 2010, WHO 2012). For the occurrence of the metastatic process, tumor cells have to shed from tumor masses, gain access to the bloodstream, and finally bind and traverse the vascular endothelium and basement membrane as well as survive and grow in secondary tissues. N2 neutrophils play important roles in all the aforementioned steps. During their trafficking within the tissue into tumor sites, neutrophils use proteases capable of digesting the extracellular matrix, which not only generates venues for spread of malignant cells but also makes possible detachment of tumor cells from tumor masses (Welch et al. 1989; Van Coillie et al. 2001; Piccard et al. 2012). Once in the bloodstream, tumor cells are passively transported until their extravasation to colonize tissues. This event involves complex mechanistic pathways and interactions between tumor cells, neutrophils, and endothelial cells. First, neutrophils interact with endothelial cells as discussed in Sect. 18.1). Briefly, integrin and integrin-like proteins, such as ICAM-1 and ICAM-2 on endothelial cells and β_2 integrins on the neutrophil surface, are important for this interaction. Then, circulating tumor cells are captured by adhered neutrophils. This appears to be mediated almost entirely by Mac-1 as well as ICAM-1 and $\alpha_v\beta_3$ integrin expressed in the surface of neutrophils and tumor cells, respectively (Huh et al. 2010; Spicer et al. 2012). Entrapped tumor cells produce CXCL8, which increases the expression and activity of the adhesion molecules and attracts other neutrophils to the site of tumor invasion (Huh et al. 2010; Piccard et al. 2012), probably allowing the capture of a higher number of tumor cells. Another effect of CXCL8 production is the activation of the neutrophils that are in close contact with tumor cells, leading to the secretion of proteases that will degrade the endothelial basement membrane and tissue extracellular matrix. Therefore, neutrophils may serve as a carrier to assist tumor cell transendothelial migration, facilitating extravasation (Wu et al. 2001). Altogether, this scenario creates favorable conditions for tumor cell invasion (Welch et al. 1989; Fridlender and Albelda 2012; Piccard et al. 2012). However, the influence of neutrophils in the metastatic processes must be

interpreted carefully as neutrophils that acquire a high cytotoxic phenotype (probably polarized into the N1 phenotype) were shown to protect an organism against tumor cell seeding and colonization of other tissues (Granot et al. 2011; Lopez-Lago et al. 2013).

18.4 Concluding Remarks and Future Perspectives

The concept that tumors may originate in areas of chronic inflammation dates from 1863, when the pathologist Rudolf Virchow observed the presence of leukocyte infiltrates in neoplastic tissues and suggested a connection between inflammation and cancer. Previous studies showed that most of the leukocyte infiltrates associated with various tumor types are composed of neutrophils. Historically, the actions of neutrophils in tumor biology was thought to be restricted to fight tumor growth, as these cells have a high cytotoxic profile. However, the observations that neutrophils may play the opposite role in tumor development by supporting the onset, growth, and even metastasis of tumors in different models of cancer (including human patients) started an intense debate permeated by controversies about the real participation of neutrophils in cancer.

The discovery that neutrophils can assume an antitumoral or a protumoral phenotype depending on the tumor microenvironment was a breakthrough in the field of tumor biology. In addition to allowing the critical interpretation of the previous results in the light of the putative phenotype present in different cancer models, the results also brought about a completely new panorama for cancer treatment. The pharmacological management of specific factors that would result in the polarization of neutrophils into a protumoral phenotype may be an interesting therapeutic approach to control or reduce tumor growth or even abrogate this disease. Nonetheless, methods to locally control this polarization in well-established tumors moving the balance toward an antitumoral fate would also be an important strategy in clinical practice.

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Chapter 19
MicroRNA Control of Apoptotic Programs in Cancer

Reena V. Kartha and Subbaya Subramanian

Abstract MicroRNAs (miRNAs) are small noncoding regulatory RNAs that are involved in diverse cellular functions, including control of cell growth, differentiation, and apoptosis. Expression of miRNAs is deregulated in most cancers and plays a significant role in all aspects of cancer development. Evasion of apoptosis, or programmed cell death, is one of the hallmarks of cancer. Apoptosis is a well-orchestrated, conserved, irreversible cellular mechanism that balances the effects of cell proliferation and cell death. Although the complex role of miRNAs in the regulation of cellular apoptosis is not fully understood, recent data strongly suggest that miRNAs play a key role in regulating apoptosis and downstream signaling pathways associated with it. Many of the apoptotic genes, irrespective of their proapoptotic or antiapoptotic functions, are regulated by miRNAs. Thus, the miRNAs involved in apoptotic pathways can be broadly classified as proapoptotic and antiapoptotic miRNAs. In this chapter, we discuss the emerging role of miRNAs in regulating cellular apoptosis in cancer.

Keywords Cancer • Apoptosis • MicroRNAs • Apoptotic pathways • Proapoptosis • Antiapoptosis • Gene regulation • Signaling pathways

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19.1 MicroRNA Biogenesis

MicroRNAs (miRNAs) are evolutionarily conserved small noncoding RNAs that posttranscriptionally regulate gene expression. They are located in both introns and exons of protein-coding genes and also occur in the intergenic regions. In general, miRNAs are transcribed by RNA polymerase II as primary transcripts (pri-miRNAs) in the nucleus. Pri-miRNA transcripts may contain multiple miRNAs (polycistronic) or single miRNA (monocistronic) (Altuvia et al. 2005). The pri-miRNAs are processed by Drosha, an RNase III enzyme, to form a stem loop structure, approximately 70 nucleotides long, called precursor miRNA (pre-miRNA). The pre-miRNAs are then exported by exportin 5 to the cytoplasm to be processed as mature double-stranded miRNAs. In the cytoplasm, the pre-miRNAs are processed by another RNase III enzyme, Dicer, and bound to miRNA-induced silencing complex (miRISC) to form the mature miRNAs, 18–24 nucleotides in length. The key proteins in miRISC are argonaute 2 and transactivation-responsive RNA-binding protein. In miRISC, the active strand is retained and the star or passenger strand is selectively degraded. The "seed" sequence in the active strand directs miRISC to complementary sites (typically in the 3′ untranslated regions, UTRs) in the target messenger RNA (mRNA) transcripts and negatively regulates gene expression (Lee et al. 1993; Eulalio et al. 2008; Bartel 2009). Posttranscriptional regulation or mRNA degradation of the target is determined on the basis of the miRNA complementarity with the target sequences (Yekta et al. 2004; Eulalio et al. 2008). The miRNAs that bind to mRNA targets with imperfect complementarity regulate the target gene at the level of protein translation. On the other hand, miRNAs that bind to their mRNA targets with perfect complementarity induce degradation (Yekta et al. 2004; Eulalio et al. 2008). A schematic representation of miRNA biogenesis is shown in Fig. 19.1.

19.2 MicroRNA-Mediated Gene Regulation

More than 1,400 miRNAs have been identified in humans (Griffiths-Jones et al. 2008). Each miRNA can potentially regulate the expression of several hundred mRNA targets (Friedman et al. 2009), and these target genes are generally conserved across species (Stark et al. 2003). Effective binding of miRNA to its target depends on several characteristic features that include (1) perfect complementarity to the "seed region" of the miRNA, (2) phylogenetic conservation of the target sequence, and (3) the availability and position of the miRNA binding site in the 3′ UTR (Grimson et al. 2007; Bartel 2009). It has been predicted that over 50% of protein-coding transcripts in mammalian genomes are regulated by miRNAs (Lewis et al. 2005; Friedman et al. 2009). In general, miRNAs interact with the 3′ UTR of their target gene. Recent reports, however, have demonstrates that miRNA targeting can also occur in the 5′ UTR, open reading frames, and promoter regions (Kloosterman et al. 2004; Lytle et al. 2007; Place et al. 2008;

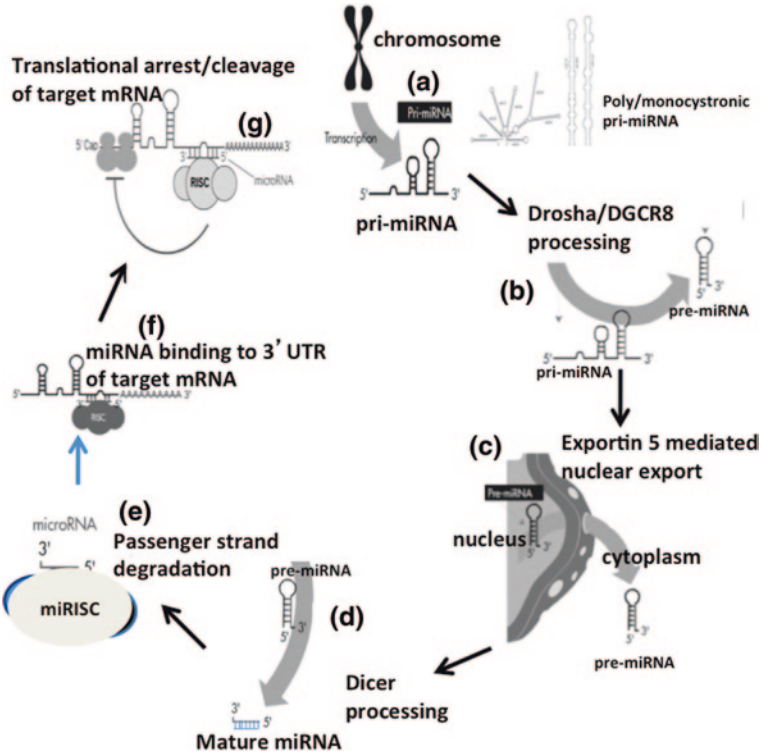

Fig. 19.1 Biogenesis of microRNAs (*miRNA*): *(a)* transcription of primary miRNAs (*pri-miRNA*) in the nucleus; *(b)* pri-miRNA is processed by Drosha and DGCR8 to form a precursor miRNA (*pre-miRNA*); *(c)* pre-miRNA is exported to the cytoplasm by exportin 5; *(d)* final processing of pre-miRNA to mature miRNA by Dicer; *(e)* incorporation of mature miRNA duplex into miRNA-induced silencing complex (*miRISC*); *(f)* binding of miRISC to the messenger RNA (mRNA) target guided by the miRNA seed sequence; *(g)* regulation of protein translation or degradation of the mRNA transcript based on the complementarity of the miRNA to the target sequence. *UTR* untranslated region. (Adapted from Subramanian and Steer 2010)

Lee et al. 2009a). For example, miRNAs such as miR-10a and miR-346 can bind to the 5′ UTR of the target genes and enhance translation of ribosomal protein mRNAs (Orom et al. 2008) and receptor-interacting protein (Tsai et al. 2009), respectively.

In mammals, miRNA-mediated gene regulation is generally accomplished by imperfect base pairing of miRNAs to target genes. Several mechanisms, such as inhibition of translation before initiation (Pillai et al. 2005; Mathonnet et al. 2007) and after initiation (Petersen et al. 2006) and translational repression of the target gene, have been described. Cytoplasmic structures such as processing bodies have been implicated in miRNA regulation (Liu et al. 2005). These processing bodies restrict the access of protein translational machinery to mRNA by storing the repressed mRNA (Eulalio et al. 2008).

Understanding miRNA–mRNA association is a complex task. A single miRNA can regulate or fine-tune multiple genes. On the other hand, depending on the number of miRNA complementary sites in the 3′ UTR of the target gene, an mRNA can be regulated by one or more miRNAs. For example, the 3′ UTR of RAS transcript contains multiple binding sites for let-7 (Johnson et al. 2005). Several miRNA target prediction programs such as Targetscan, miRanda, miRgen, miRecords, and miRdb are available to identify potential target genes. These target-prediction programs use unique algorithms and consider several factors, such as conservation of miRNA binding sites, mRNA secondary structures, and seed sequence match, to identify potential targets (Bartel 2009). In addition, several bioinformatics tools which can correlate miRNA and mRNA expression levels are also available to better understand the miRNA gene regulatory networks (Creighton et al. 2008).

19.3 Overview of Apoptosis

Apoptosis is a highly regulated cellular process which can be divided into three distinct phases: initiation, integration/decision, and execution/degradation (Kroemer et al. 1997). Apoptosis can be mediated by several molecular pathways (Hotchkiss et al. 2009). The most prominent and best characterized are the intrinsic pathway, involving mitochondria (Shi 2002), and the extrinsic pathway, which is activated by death receptors at the cellular membrane (Ashkenazi 2002) (Fig. 19.2). Although generally independent, these two apoptotic pathways interact via molecular cross talk of key proteins common to both.

Death receptors are type 1 transmembrane proteins belonging to the tumor necrosis factor (TNF) receptor superfamily (Locksley et al. 2001), including TNF receptor 1, Fas (CD95/Apo-1), TNF-related apoptosis-inducing ligand (TRAIL) receptors 1 and 2, and death receptors 3 and 6. The members of the TNF receptor family share similar cysteine-rich extracellular domains (N-terminal) and have a cytoplasmic domain of about 80 amino acids called the “death domain” (C-terminal) (Ashkenazi and Dixit 1998), which plays a critical role in transmitting the death signal from the cell surface to intracellular signaling pathways. Once activated, death receptors induce the cleavage and activation of procaspases 8 and 10 via dimerization of the death effector domain. In type I cells such as thymocytes, caspases 8 and 10 directly activate downstream caspases, such as caspase 3 or caspase 7 (Peter and Krammer 2003). In type II signaling cells, such as hepatocytes, progression of the apoptotic cascade occurs by targeting mitochondria (Li et al. 2002). In this case, caspase 8 cleaves inactive cytoplasmic Bid, exposing an active truncated fragment (Scaffidi et al. 1998). Once activated, tBid induces conformational changes in proapoptotic Bax and Bak and subsequent translocation to the mitochondria where they induce release of apoptogenic factors, such as cytochrome *c* (Eskes et al. 2000).

Typically, antiapoptotic members of the Bcl-2 family (e.g., Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, and A1) are integral membrane proteins, usually localized in the mitochondrial outer membrane. In contrast, proapoptotic members of the Bcl-2 family, such as Bax,

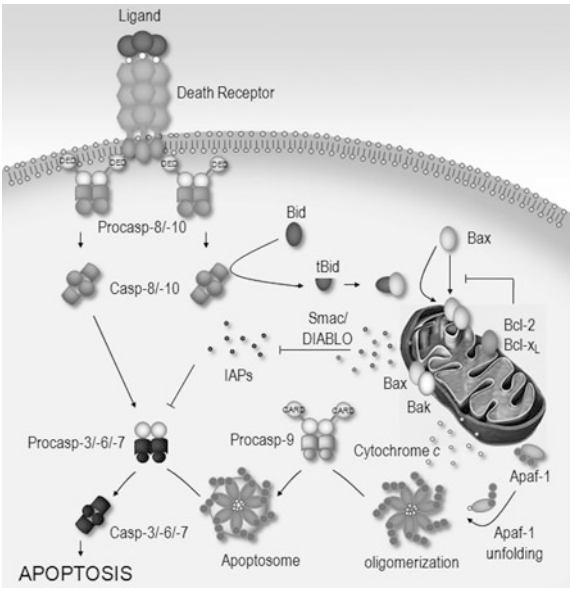

Fig. 19.2 Overview of extrinsic and intrinsic apoptotic pathways. In the extrinsic pathway, death receptors recruit adaptor proteins and activate caspase 8 and 10 after interacting with their ligands. These initiator caspases then cleave effector caspases 3, 6, and 7, which activate key downstream targets and execute the apoptotic process. In the mitochondrial intrinsic pathway, death stimuli target mitochondria either directly or through transduction by proapoptotic Bax and Bak. Mitochondria then release cytochrome *c* and Smac/DIABLO, among other apoptogenic factors. Smac/DIABLO inhibit inhibitors of apoptosis (*IAPs*), which in turn bind and inhibit caspases. Cytochrome *c* induces oligomerization of apoptotic protease-activating factor 1 (*Apaf-1*), which recruits and activates procaspase 9 to caspase 9, which then activates effector caspases. The cross talk between both pathways is mediated by Bid, which is truncated and activated by caspase 8. *DED* death effector domain, *casp* caspase, *procasp* procaspase, *tBid* truncated Bid. (Adapted from Subramanian and Steer 2010)

are located in the cytosol as inert forms or inactive precursors (Gross et al. 1999). Truncated Bid can also inhibit antiapoptotic proteins, such as Bcl-2, (Kim et al. 2000), or even directly permeabilize the mitochondrial outer membrane (Goonesinghe et al. 2005), promoting the release of cytochrome *c*, and activating downstream caspase 3. Intriguingly, antiapoptotic members of the Bcl-2 family, such as Bcl-2 and Bcl-x_L, interact with Bax, preventing pore formation and release of apoptogenic proteins, thereby mitigating apoptosis progression (Antonsson et al. 1997).

Mitochondria also play a critical role in the regulation of cell death. Following an apoptotic stimulus, such as oxidative stress, DNA damage, or protein misfolding, increased calcium levels permeabilize the mitochondrial membrane, causing release of apoptogenic factors (Ricci et al. 2003). Opening of the mitochondrial permeability transition pore and loss of inner mitochondrial transmembrane potential result in the cytosolic release of two main groups of normally sequestered

proapoptotic proteins from the intermembrane space. The first group consists of cytochrome *c*, second mitochondria-derived activator of caspases/direct inhibitor of apoptosis (IAP)-binding protein with low pI (Smac/DIABLO), and the serine protease HtrA2/Omi. These proteins activate a family of death-inducing cysteine proteases, termed caspases, which cleave a number of cellular substrates. Once in the cytosol, cytochrome *c* oligomerizes with the apoptotic protease-activating factor 1 (Apaf-1) in the presence of dATP, recruiting procaspase 9 to form the apoptosome. This complex formation results in cleavage and activation of caspase 9, which in turn cleaves and activates other caspases that function as downstream effectors of the cell death program (Zou et al. 1999). Smac/DIABLO (Du et al. 2000) and HtrA2/Omi (Martins et al. 2002) are both proteolytically processed within the intermembrane space to yield mature polypeptides, which in turn are reported to promote apoptosis by inhibiting members of the IAP protein family.

The second group of proteins released includes apoptosis-inducing factor, endonuclease G, and DNA fragmentation factor 40 or caspase-activated DNase (Elmore 2007). During apoptosis, the mitochondrial localization sequence of apoptosis-inducing factor is cleaved and gives rise to a mature form of the protein that is translocated from the mitochondria to the cytosol and subsequently to the nucleus. In the nucleus, it promotes chromatin condensation and large-scale DNA fragmentation. A similar role is played by endonuclease G, a mitochondria-specific endonuclease that translocates to the nucleus during apoptosis to cleave single-stranded and double-stranded DNA and RNA (Li et al. 2001). After caspase-activated DNase has been released from the mitochondrion, it translocates to the nucleus after cleavage by caspase-3, leading to DNA nucleosomal fragmentation and advanced chromatin condensation (Enari et al. 1998).

Caspases are critical downstream executioners of the apoptotic cascade (Thornberry and Lazebnik 1998). They are prominent players in the apoptosis process owing to their role in abrogating survival pathways and activating downstream events that are responsible for cell dismantling and death. These proteins, synthesized as inactive precursor forms, undergo a process of activation during apoptosis, and are classified as either initiator caspases (e.g., caspases 2, 8, 9, and 10) or effector caspases (e.g., caspases 3, 4, 5, 6, 7, 11, 12, and 13) (Shi 2002). Initiator caspase 9 activates effector caspase 3, which in turn activates downstream factors that ultimately result in cell death. Caspase 3 is responsible for the proteolytic cleavage of poly(ADP-ribose) polymerase (Rosen and Casciola-Rosen 1997), among many other substrates. The IAP proteins are the primary inhibitors of caspase activation. (Crook et al. 1993). The transcription factor p53 (Erster et al. 2004) and CmrA (a cytokine response modifier gene) (Ray et al. 1992) can also regulate the activation of caspases. Cells can also undergo apoptosis through caspase-independent pathways, involving several other proteases such as cathepsins, calpains, and granzymes.

The transcription factor E2F1 regulates the cell cycle and induces apoptosis by inhibiting antiapoptotic signals, including nuclear factor κB (Phillips et al. 1999). Further, E2F1 induces p53 activity through an indirect mechanism (Bates and Vousden 1999). In fact, it was reported that E2F1 can stabilize p53 via induction of the human tumor suppressor protein $p14^{ARF}$ (Bates et al. 1998). Protein p53

functions as a sequence-dependent transcription factor that regulates diverse groups of genes under different stress conditions, ultimately leading to growth arrest, senescence, and apoptosis. The tumor suppressor is typically maintained at a low level by a negative-feedback loop in which it induces the transcription of Mdm-2, which in turn binds to p53 and mediates its degradation. The mechanism by which p53 signals members of the Bcl-2 family of proteins is not entirely understood. However, it has been shown that proapoptotic members of the Bcl-2 family, such as Bax, Noxa, and Puma, are transcriptional targets of p53 (Miyashita and Reed 1995; Schuler and Green 2001; Thornborrow and Manfredi 2001). In addition, p53 downregulates the antiapoptotic member Bcl-2, thus inducing the release of apoptogenic factors from the mitochondria (Miyashita et al. 1994).

Finally, there are several well-characterized antiapoptotic pathways, including phosphatidylinositide 3′-kinase survival signaling. Once activated, phosphatidylinositide 3′-kinase catalyzes the generation of 3′-phosphorylated phospholipids that activate a growing number of cellular intermediates, including the serine/threonine protein kinase Akt, which suppresses cell death. In fact, it has been shown that Akt can induce phosphorylation of proapoptotic Bad, resulting in 14-3-3 binding and sequestration from Bcl-x_L (Datta et al. 1997; del Peso et al. 1997). In contrast, the loss of these survival signals promotes dephosphorylation of Bad and its translocation to the mitochondria (Wang et al. 1999). Akt can also act as an important signaling intermediate upstream of survival gene expression, which is dependent on nuclear factor κB (Kane et al. 1999).

Another aspect of apoptotic pathways that is not fully understood is the contribution of miRNAs in regulating or fine-tuning the apoptotic machinery and the genes associated with it. We now know that miRNAs play a major role in regulating apoptosis in cancer and other developmental processes. To identify the miRNA–mRNA regulatory network, we must first delineate the mechanisms by which miRNAs function. In humans, it is estimated that more than 50 % of coding gene transcripts are targeted by miRNAs. To date, over 50 miRNAs are recognized to play a role in apoptosis. The key genes involved in apoptotic pathways can be potential targets of one or more of these miRNAs. There are numerous interacting pathways that are involved in the regulation of apoptosis, and miRNAs appear to be involved at every intersection (Yang et al. 2009).

19.4 Transcription Factor p53 and MicroRNA Maturation

A major regulator of apoptosis and senescence is the transcription factor p53. In cancer, p53 is inactivated either by mutations or through other molecular mechanisms. Mutations in p53 predominantly occur in the DNA-binding domain by which it regulates many apoptotic proteins. Active p53 DNA-binding domain has also been implicated in the maturation of miRNAs (Suzuki et al. 2009). It was shown that, in response to DNA damage, the DNA-binding domain of p53 interacts with the Drosha complex preferably at the p68 RNA-helicase component of

the complex to facilitate the processing of selected pri-miRNAs to pre-miRNAs. However, mutations in the DNA-binding domain affect the p53 interaction with the Drosha complex, thereby directly affecting the processing of miRNAs such as miR-16 and miR-143, which suppress cell proliferation. Further, p53 regulates apoptosis by modulating the levels of miRNAs (He et al. 2007a), and its transcriptional network includes members of the miR-34 family (He et al. 2007a), miR-215, and miR-192 (Georges et al. 2008).

19.5 MicroRNAs and Cellular Apoptosis

Apoptosis is one of the major cell regulatory pathways that is affected in cancer. An increasing number of studies clearly show the link and role of miRNAs in apoptosis, especially in cancer development (Calin and Croce 2006). In fact, miR-14 and *batman* were the first miRNAs to be associated with apoptotic function in *Drosophila* (Brennecke et al. 2003; Xu et al. 2003). The miRNAs involved in tumor onset and progression are collectively called oncomiRs (Esquela-Kerscher and Slack 2006). More than 50 miRNAs have been implicated in the regulation of apoptosis (Table 19.1). MicroRNAs (miRNAs) are generally downregulated in tumor conditions compared with normal tissues (Lu et al. 2005). Defects in the miRNA biogenesis machinery can cause deregulation of miRNAs and promote tumor formation (Kumar et al. 2007). For instance, loss or inactivation of Dicer, an endonuclease required for miRNA maturation, leads to decreased levels of miRNAs, such as let-7a, miR-16, and miR-21, which are implicated in apoptosis (Ghodgaonkar et al. 2009). Further, the cellular apoptotic machinery can also regulate Dicer function, resulting in apoptosis (Matskevich and Moelling 2008). Both proapoptotic and antiapoptotic genes are potentially regulated by miRNAs. Since miRNAs can regulate hundreds of gene targets, a single miRNA can function as proapoptotic and/or antiapoptotic depending on the cellular context and the target gene.

19.5.1 Proapoptotic MicroRNAs

In cancer cells, there is a predominant downregulation of proapoptotic miRNAs, which downregulates antiapoptotic genes. Recently, in a screen for miRNAs that modulate p53 activity, miR-29 family members (miR-29a, miR-29b, and miR-29c) were observed to induce apoptosis in a p53-dependent manner (Park et al. 2009b). These miRNAs activate p53 by targeting *p85α* and *CDC42* (Park et al. 2009b). Further studies have also shown the miR-29 family targets Mcl-1 in cholangiocarcinoma cells (Mott et al. 2007). In addition, miR-29 also targets the DNA methylating genes *DNMT3A* and *DNMT3B* and, thus, can counteract CpG methylation, leading to the expression of proapoptotic genes that may have been silenced by methylation (Fabbri et al. 2007). CDC42, which negatively regulates p53, is

Table 19.1 MicroRNAs implicated in apoptotic function

MicroRNAs	Cancer type	Target genes implicated in apoptosis
Proapoptotic		
let-7 family	Lung, ovarian, liver, breast, lymphoma	*RAS*, *HMGA2*, *MYC*
miR-1	–	*HSP60*, *HSP70*
miR-10b	Glioma	*BIM*
miR-29a, miR-29b, miR-29c	Colon, CLL, lung	*MCL1*, *DNMT3A*, *DNMT3B*, *CDC42*, p85α, *BCL2*
miR-31	Breast	*BIM*
miR-34a, miR-34b, miR-34c	Colon, MPNST, neuroblastoma, lung, prostate, pancreatic, leukemia	*SIRT1*, *CDK4*, *E2F3*
miR-1516	CLL, pancreatic, prostate, gastric	*BCL2*
miR-101	Hepatoma	*MCL1*
miR-122	HCC	*BCLW*
miR-133b	Lung	*MCL1*, *BCL2L2*
miR-137	CRC	*CDC42*
miR-143	Osteosarcoma	*BCL2*
miR-145	Breast, colon	*RTKN*, *PAK4*
miR-150	Lymphoma	*DKC*, *AKT*
miR-153	Glioblastoma	*MCL1*, *BCL2*
miR-181a, miR-181b	Leukemia, multiple cancers	*BCL2*
miR-185	CRC	*CDC42*
miR-193b	HCC	*MCL1*
miR-195	CRC	*BCL2*
miR-203	Colon	*BAX*, *AKT*
miR-205	Melanoma	*E2F1*, *E2F5*
miR-212	NSCLC	*PED*
miR-224	HCC	*API5*
miR-375	Gastric carcinoma	14-3-3
miR-491	CRC	*BCLXL*
miR-512-3p	HCC	cFLIP
miR-1204	Cancer cell lines	p53
14q32miRs	Osteosarcoma	c-*MYC*
Antiapoptotic		
miR-17-92	Colon, prostate	*E2F*, *BIM*, p21
miR-17-5p	Cervical	*TP53INP1*
miR-21	Breast, cervical, colon, gastric, glioblastoma, HCC, prostate	*PDCD4*, *PTEN*/*AKT*, *TPM1*, *RECK*, *FASL*
miR-24, miR-24a	Gastric, cervical, prostate	*FAF1*, *XIAP*, caspase 9, *APAF1*
miR-25	Esophageal	*BIM*
miR-32	Prostate	*BIM*
miR-92	Colon	*BIM*
miR-93	Neuroblastoma	p21
miR-96	Breast	Caspase 3, *FADD*

(continued)

Table 19.1 (continued)

MicroRNAs	Cancer type	Target genes implicated in apoptosis
miR-106b-25	Colon, gastric, multiple myleloma, neuroblastoma, prostate	p21, *BCL2L11*
miR-125b	Prostrate, breast carcinoma, liver, lung, CRC	*BAK1*, *MCL1*, *BCLW*, *IL6R*
miR-128	Kidney cells	*BAX*
miR-133	–	Caspase 9
miR-143	Colon	*ERK5*
miR-145	Multiple cancer cell lines	*DR4*, *DR5*
miR-155	Colon, lung, pancreatic, Burkitt lymphoma, Hodgkin lymphoma	*TP53INP1*
miR-181a	Non-Hodgkin lymphoma	*BIM*, caspase 3, *FADD*
miR-182	Breast	Caspase 3, *FADD*
miR-183	Synovial sarcoma, rhabdomyosarcoma, colon	*EGR1*
miR-186*	Human lung adenocarcinoma	Caspase 10
miR-199a	Gastric	*SMAD4*
miR-200c	Colon	FAP-1
miR-206	Breast	Estrogen receptor α
miR-214	Ovarian	*PTEN*
miR-216	Multiple cancer cell lines	*DR4*, *DR5*
miR-221/222	Melanoma, NSCLC, breast, CLL, glioblastoma, thyroid papillary carcinoma, gastric, liver	*CDKN1B* (p27), c-*KIT*, *CDKN1C* (p57), *PUMA*
miR-296-5p	Liver	*PUMA*
miR-330	Prostate	*E2F1*
miR-378	Cardiac	Caspase 3
miR-483-3p	Liver	*PUMA*

CLL chronic lymphocytic leukemia, *CRC* colorectal cancer, *NSCLC* non-small-cell lung cancer, *HCC* hepatocellular carcinoma, *MPNST* malignant peripheral nerve sheath tumor

also targeted by miR-137 and miR-185 and inhibits proliferation and invasion in colorectal cancer cells (Liu et al. 2011a, b). Similarly, p53 regulating p85α is also targeted by miR-376a in hepatocellular carcinoma (HCC); however, significant downregulation of miR-376a in HCC may result in the negative regulation of p53 mediated by p85α (Zheng et al. 2012). The stability of p53 is regulated by the 14-3-3ζ isoform in mammary epithelial cells (Danes et al. 2008). In gastric carcinomas, 14-3-3ζ was observed to be targeted by miR-375, and its ectopic expression reduced cell viability via a caspase-mediated apoptosis pathway (Tsukamoto et al. 2010).

It has been shown that p53 activation induces expression of miRNAs, including tumor suppressor miRNAs such as let 7a, miR-15a/16, and miR-34a; miR-34a is a tumor suppressor and a proapoptotic miRNA directly transactivated by p53 (Chang et al. 2007; Raver-Shapira et al. 2007; Craig et al. 2012). Among the miR-34 family,

miR-34a and miR-34c share an "identical seed sequence." Both play a critical role in androgen-receptor-dependent p53-mediated apoptosis in prostate cancer (Rokhlin et al. 2008). In humans, loss of expression of miR-34a , which is located in chromosome band 1p36.23 is common in tumors as either this region is frequently deleted in several tumor conditions, including neuroblastomas, or the miR-34a promoter is subject to inactivation by CpG methylation (Lodygin et al. 2008). Earlier, we showed that overexpression of miR-34a induces apoptosis in malignant peripheral nerve sheath tumor cell lines (Subramanian et al. 2010). Targets of miR-34a include genes such as *CDK4*, *CCND1*, *CCNE2*, *MET*, *MYCN*, *SIRT1*, *BCL2*, *E2F1*, *E2F3*, and *E2F5*, all of which are involved in apoptosis and cell proliferation (Welch et al. 2007; Wei et al. 2008). However, in a genome-wide RNA interference screen, only *CDK4* had a unique function in apoptotic signal transduction among the cyclin-dependent kinase genes (Ovcharenko et al. 2007). Further, miR-34a is also known to induce apoptosis by inhibiting *SIRT1*, which leads to expression of p21 and Puma (Yamakuchi et al. 2008). In a recent study, nicotinamide treatment in chronic lymphocytic leukemia (CLL) was shown to significantly induce miR-34 levels and repress *SIRT1*, suggesting a therapeutic potential for nicotinamide, a safe, inexpensive novel drug as adjunctive therapy in CLL patients with wild-type p53 (Audrito et al. 2011).

Both miR-15 and miR-16 are considered proapoptotic miRNAs as they regulate antiapoptotic factor Bcl-2. These miRNAs can induce apoptosis and negatively regulate cell growth and the cell cycle when exogenously expressed in CLL and other tumor cell lines (Cimmino et al. 2005; Linsley et al. 2007). In CLL, the miR-15-16 cluster is frequently deleted or downregulated, which can induce overexpression of Bcl-2 (Cimmino et al. 2005). The antiapoptotic gene *BCL2* is upregulated in several human cancer types, including Hodgkin lymphoma, B-cell lymphoma, and breast cancer. Hence, miR-15-16-mediated control of Bcl-2 can be deregulated in these tumors. In addition to deletion of miR-15-16 clusters in CLL, a mutation in these miRNA genes is also described that leads to downregulation of this cluster (Calin et al. 2005). Further, miR-153 is yet another brain-specific miRNA that targets Bcl-2. It was significantly downregulated in glioblastoma compared with nonneoplastic brain tissue (J. Xu et al. 2010). Several other miRNAs, such as miR-143, miR-181a, miR-181b, and miR-195 have been shown to regulate Bcl-2 expression in diverse cancers. Of these, miR-181a has also been shown to negatively regulate RalA in leukemic cells and promotes apoptosis (Fei et al. 2012). Likewise, many of the miRNAs mentioned here target more than one antiapoptotic gene, and one gene can be targeted by more than one miRNA. Thus, miRNAs regulate apoptosis via more than one pathway (Table 19.1). For instance, miR-153 targets both Bcl-1 and Mcl-1 (J. Xu et al. 2010). In contrast, Mcl-1 is targeted by miR-15a, miR-16-1, the miR-29 family, miR-101, miR-133b, miR-153, and miR-193b in various cancers. Similarly, E2F1 is targeted by proapoptotic miRNAs: miR-93, miR-106b, miR-149*, and miR-330 (Petrocca et al. 2008; Lee et al. 2009b; Lin et al. 2010). In fact, the miR-106b-25 cluster, which is upregulated in a subset of human gastric tumors, is activated by E2F1. In turn, miR-106b and miR-93 regulate E2F1 expression, establishing a miRNA-directed negative-feedback loop in gastric cancer (Petrocca et al. 2008).

The 11 member let-7 family of miRNAs is a highly conserved miRNA family implicated in cellular development. Let-7 is regulated by LIN28, an RNA-binding protein. Binding of LIN28 to the primary let-7 transcript blocks Drosha-mediated processing of the primary transcript (Viswanathan et al. 2008). Let-7a regulates HMGA2 and RAS oncogene expression, thus behaving as a tumor suppressor miRNA (Lee and Dutta 2007; Mayr et al. 2007). Moreover, the let-7 family of miRNAs was found to be downregulated in human hepatoma cells (Huh7) and has a putative target site in Bcl-x_L mRNA. Overexpression of let-7c and let-7g decreased Bcl-x_L expression and enhanced sorafenib-induced apoptosis (Shimizu et al. 2010) in HCC. Conversely, let-7 also specifically targets caspase 3 and regulates apoptosis in an antiapoptotic manner (Tsang and Kwok 2008).

There are also examples of miRNAs targeting other genes in the apoptotic pathway. For example , miR-143 is known to target ERK5 and promote apoptosis by a FasL-mediated feedback loop in human leukemia cells (Akao et al. 2009). Further, miR-491 directly targets Bcl-x_L in colorectal cancer cell lines (Nakano et al. 2010). PED/PEA-15 is an antiapoptotic protein that interferes with both intrinsic and extrinsic apoptotic pathways, inhibiting the formation of death-inducing signaling complex and caspase 3 activation. Also, miR-212 is a negative regulator of PED/PEA-15 in non-small-cell lung cancer and increases TNF-related apoptosis (Incoronato et al. 2010). Fas-associated phosphatase 1, which inhibits apoptosis induced by Fas, is a direct target of miR-200c (Schickel et al. 2010). Cellular FLICE-inhibitory protein (c-FLIP) is another protein that inhibits recruitment of caspase 8, and recently miR-512-3p was demonstrated to negatively regulate c-FLIP in HCC cells (Chen et al. 2010). Further, miR-224 targets apoptosis inhibitor 5, which is a suppressor of E2F1-dependent apoptosis in HCC (Wang et al. 2008b). There is also induction of apoptosis by miR-145 in MCF7 breast cancer cells by targeting RTKN (Wang et al. 2009) and PAK4 via the mitogen-activated protein kinase pathway in colon cancer (Wang et al. 2012). Finally, miR-101 expression in hepatoma cell lines sensitizes both serum starvation and drug-induced apoptosis by targeting Mcl-1 (Su et al. 2009).

Modulation of proapoptotic miRNAs, such as miR-1, can be achieved by using small-molecule inhibitors. In lung cancer cells, the reduced levels of miR-1 can be reactivated by histone deacetylase inhibitor, leading to reduced expression of MET and PIM1, thus reversing the clonogenic survival and tumor formation of lung cancer cells in nude mice (Nasser et al. 2008). Further, miR-206 is also predominantly expressed in muscle tissues and is implicated in rhabdomyosarcoma, a skeletal muscle tumor (Taulli et al. 2009). Overexpression of miR-206 in human cancer cells decreased the levels of both Notch3 mRNA and protein and blocked the antiapoptotic activity of Notch3, leading to induced apoptotic cell death (Song et al. 2009). Ectopic expression of miR-1 and miR-206 shows significant downregulation of PAX3 and cyclin D2 protein expression in embryonal rhabdomyosarcoma (Li et al. 2012).

Recently, we identified a subset of miRNAs from the human 14q32 locus (miR-382, miR-369-3p, miR-544, and miR-134) that could potentially target *cMYC* (Thayanithy et al. 2012) in osteosarcoma cells. Restoring the expression of these four 14q32 miRNAs decreased cMYC levels and synergistically induced apoptosis

in Saos2 cells. Further, exogenous expression of 14q32 miRNAs in Saos2 cells significantly downregulated miR-17-92, a transcriptional target of *cMYC*. The proapoptotic effect of 14q32 miRNAs in Saos2 cells was reversed either by overexpression of *cMYC* complementary DNA without the 3′ UTR or by the miR-17-92 cluster. Together, our data support a model where the deregulation of a network involving 14q32 miRNAs, *cMYC*, and miR-17-92 could contribute to osteosarcoma pathogenesis (Thayanithy et al. 2012).

19.5.2 Antiapoptotic MicroRNAs

Although miRNAs that target the proapoptotic genes are considered antiapoptotic, they can also prevent apoptosis by promoting cell proliferation. As an example, MYC is a major regulator of cell proliferation and apoptosis. MYC can transactivate miRNAs in the miR-17-92 cluster that can potentially target E2F1, thus forming a negative-feedback loop (O'Donnell et al. 2005). The miR-17-92 cluster (locus in chromosome band 13q31.3) is composed of seven miRNAs: miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1(He et al. 2005). Mathematical modeling predicts that members of the miR-17-92 cluster have the same properties as E2F/MYC, thus acting either as tumor suppressors or oncogenes depending on their expression levels (Aguda et al. 2008). Inhibition of miR-17-5p and miR-20a with antisense oligonucleotides induces apoptosis in lung cancer cells (Matsubara et al. 2007); miR-17-5p regulates *TP53INP1* in cervical cancer (Wei et al. 2012), whereas this gene is targeted by miR-155 in pancreatic ductal carcinoma (Gironella et al. 2007). It has been shown that overexpression of NKX2.5 in T cell acute lymphoblastic leukemia cell lines results in the activation of miR-17-92, and a corresponding decrease in the expression of E2F1 (Nagel et al. 2009). Recently, it was shown that silencing MYC in multiple myeloma cell lines downregulates the expression of several components of the miR-17-92 cluster and induces apoptosis (Kanzaki et al. 2011). Further, hypoxia-inducible factor 1α was identified as another novel target for the miR-17-92 cluster in lung epithelial cells, indicating the possible existence of an intricate and finely tuned circuit involving cMYC, miR-17-92, and hypoxia-inducible factor 1α that may play a role in cancer cell proliferation under normoxia in a cellular context-dependent manner (Taguchi et al. 2008). In B cell lymphoma, miR-17-92 can also target the proapoptotic genes *CDKN1A* (p21) and BIM (Inomata et al. 2009). Knockdown of miR-17-5p with an antagomir increased BIM and p21 expression in neuroblastoma cells resistant to therapy and was sufficient to promote massive apoptosis (Fontana et al. 2008). BIM is also regulated by oncogenic miRNAs such as miR-32 (Ambs et al. 2008) in prostate cancer, by miR-25 in esophageal cancer (Kan et al. 2009), and by miR-181a in non-Hodgkin lymphoma (Lwin et al. 2010). Similarly, another miRNA cluster, miR-106b-25 can be activated by E2F1. In turn, E2F1 expression is repressed by miR-106b and miR-93 in the cluster, forming a negative-feedback loop (Petrocca et al. 2008). Upregulation of miR-106b-25 by E2F1 is detected in

most cases of gastric cancer, where these miRNAs negatively regulate the proapoptotic genes p21 and BCL2L11 in the transforming growth factor β tumor suppressor pathway, leading to tumor progression (Petrocca et al. 2008).

Caspases, which are major proapoptotic genes, are regulated by miRNAs. For instance, two miRNAs, miR-133 and miR-24a, are known to inhibit expression of caspase 9, leading to a block in mitochondrial apoptosis (Xu et al. 2007; Walker and Harland 2009). Further, miR-24a also negatively regulates proapoptotic Apaf-1 in addition to caspase 9 (Walker and Harland 2009). Caspase 10 is targeted by miR-186* in the non-small-cell lung cancer cell line. Other genes in the extrinsic cell death pathway such as Fas-associated factor 1 (*FAF1*) are directly regulated by miR-24 in gastric, cervical, and prostate cancer cells (Qin et al. 2010). Moreover, miR-24 overexpression directly modulates the *XIAP* expression level and reduces the apoptosis threshold in multiple cancer cells (Xie et al. 2012). In breast cancer cells, miR-182 and the highly homologous miR-96 were predicted to regulate FADD and caspase 3, whereas miR-145 and miR-216 were identified as putative regulators of death receptors 4 and 5 (Ovcharenko et al. 2007). Further, miR-155 also targets the 3′ UTRs of FADD and caspase 3 and downregulation of miR-155 promotes Fas-mediated apoptosis in human intervertebral disc regeneration (Wang et al. 2011). Interestingly, miR-155 has recently been shown to induce apoptosis in dendritic cells by regulating p27kip1 levels and arresting cell cycle progression (Lu et al. 2011). An oncogenic function for miR-125b has been suggested in prostate cancer since it targets the proapoptotic gene BAK1 (Shi et al. 2007). However, recently, a proapoptotic function for miR-125b was demonstrated in liver, lung, and colorectal cancer cell lines, where miR-125b targets Mcl-1, Bcl-w and IL-6 receptor (Gong et al. 2012). Another example of an antiapoptotic miRNA is miR-199a, whose expression significantly inhibited transforming growth factor β signaling by directly targeting Smad4 and plays an oncogenic role in human gastric tumorigenesis (Zhang et al. 2012). In liver carcinoma cells, *PUMA* expression was observed to be modulated by miR-483-3p (Veronese et al. 2010) and its ectopic expression can protect cells from apoptosis. *PUMA* expression is also directly regulated by miR-221/222 (Zhang et al. 2010). Yet another proapoptotic player, BAX, is under the regulation of miR-128 in human embryonic kidney cells (Adlakha and Saini 2011). Ectopic expression of miR-128 in these cells resulted in the induction of mitochondria-mediated apoptosis.

The miRNA miR-21 predominantly functions as an antiapoptotic miRNA and is consistently upregulated in many cancer cell types. It typically exerts its antiapoptotic and oncogenic function by targeting several tumor suppressor genes. It has been shown to be positively regulated by an Akt-dependent pathway and to exert its antiapoptotic effects by direct inhibition of FasL (Sayed et al. 2010). The level of miR-21 is inversely correlated with tropomyosin (*TPM1*) and phosphatase and tensin homologue (*PTEN*) expression and apoptosis in tongue squamous cell carcinomas (Li et al. 2009). Anti-miR-21 reduced proliferation and increased the amount of apoptosis of pancreatic cancer cell lines (Park et al. 2009a). With the expression of antisense miR-21 in pancreatic cancer cells, the levels of the tumor suppressor proteins PTEN and RECK increased and this resulted in cell cycle arrest (Park et al. 2009a). PTEN was also identified as a miR-21 target gene in

HCC (Meng et al. 2007). PTEN is also downregulated by miRNAs such as miR-17-5p, miR-19, miR-214, and miR-221/222 (Xiao et al. 2008; Yang et al. 2008; Chun-Zhi et al. 2010). In a recent study, we showed that elevated levels of miR-183 in colon cancer, synovial sarcoma, and rhabdomyosarcoma significantly reduce the transcript and protein levels of EGR1, a tumor suppressor and a transcription factor (Sarver et al. 2010). Since EGR1 is downregulated, its transcriptional target PTEN is also significantly affected. Blocking the activity of miR-183 using anti-miRs elevated the levels of both EGR1 and PTEN. Thus, a single miRNA, miR-183, directly and indirectly regulates two tumor suppressors, EGR1 and PTEN (Sarver et al. 2010). This study also highlights the conserved miRNA gene regulatory networks in several cancer types.

Recent studies have demonstrated that miR-21 is significantly upregulated in gastric cancer and targets *RECK*, a tumor suppressor gene (Zhang et al. 2008). Further, in human and mouse glioblastoma cells, loss of miR-21 resulted in increased apoptosis (Chan et al. 2005; Si et al. 2007). In addition, miR-21 also targets the tumor suppressor genes *PDCD4* (Asangani et al. 2008) and *TPM1* (Zhu et al. 2007). *PDCD4* is a proapoptotic gene and a negative regulator of AP1, which induces miR-21 in response to RAS activation. In fact, miR-21 overexpression has been shown to inhibit *PDCD4*-mediated apoptosis. These studies suggested that knocking down the expression of miR-21 in various cancer types might be an effective therapy for cancer. RNA-binding protein HNRPK and TAp63 are also regulated by miR-21(Papagiannakopoulos et al. 2008). It has been reported that TAp63 upregulates Bcl-2 family genes such as *BAX*, *APAF1*, and *BCL2L11* and also transactivates CD95, triggering the expression of death receptors and revealing the direct link between TAp63 and the mitochondrial pathway. MicroRNAs (miRNAs) such as miR-21, miR-130, miR-140, and miR-290 were found to be associated with rat liver mitochondria. These miRNAs were predicted to target genes associated with apoptosis, cell proliferation, and differentiation (Kren et al. 2009). Mitochondria play a role in the intrinsic apoptotic pathway and may serve as a rheostat for miRNAs that modulate the apoptotic process.

Functional genetic approaches have been used to show that miR-221 and miR-222 regulate *CDKN1B* (p27) (le Sage et al. 2007). *CDKN1B* is a tumor suppressor gene and promotes apoptosis by targeting p27 transcript. Further, miR-221/222 can promote cancer progression and behaves as antiapoptotic miRNA. The other target genes of miR-221/222 include proapoptotic *CDKN1C* (p57) (Fornari et al. 2008), *BMF* (Gramantieri et al. 2009), and estrogen receptor α (Zhao et al. 2008). Most of the known mRNA targets of miR-221/222 are proapoptotic in nature, and thus increased expression of miR-221/222 leads to cell proliferation and survival. In a recent study, miR-221 was shown to promote liver regeneration by targeting aryl hydrocarbon nuclear translocator (Arnt), which reduces apoptosis (Yuan et al. 2013). Further, miR-221/222 is also known to target cKIT (Felli et al. 2005), and activating mutations of the cKIT gene is common in gastrointestinal stromal tumors. Previously, we have shown that miR-221 and miR-222 are expressed at significantly lower levels in gastrointestinal stromal tumors (Subramanian et al. 2008). Thus, it is possible that miR-221 and miR-222 act as both antiapoptotic and proapoptotic miRNAs depending on the tumor type and its target gene.

19.5.3 Other MicroRNAs Associated with Apoptosis

A genome-wide screen for apoptotic miRNAs using miRNA inhibitors in HeLa cells revealed that a significant number of miRNAs, such as miR-95, miR-124, miR-125, miR-134, miR-144, miR-150, miR-152, miR-187, miR-190, miR-191, miR-192, miR-193, miR-204, miR-211, miR-218, miR-220, miR-296, and miR-299, caused a decrease in cell growth (Cheng et al. 2005). However, a library screen for apoptotic miRNAs in lung carcinoma cells showed inhibition of miR-7, miR-19a, miR-23, miR-24, and miR-140 and significantly decreased cell growth (Cheng et al. 2005). In a similar study, miRNAs such as let-7c, miR-10a, miR-144, and miR-150 were observed to affect the activation of the caspase cascade and negatively influence apoptosis (Ovcharenko et al. 2007). Methods to assess the direct involvement of miRNAs in apoptosis have recently been reviewed in detail (Spizzo and Calin 2009).

Several other miRNAs are also implicated in the apoptosis of cancer and/or in other developmental processes. For example, miR-1 and miR-133 are significantly expressed in muscle tissues, and are implicated in various cardiovascular diseases and in the regulation of cardiomyocyte proliferation (Tang et al. 2009). Recent evidence has shown that miR-1 and miR-133 may have opposing effects on apoptosis, with miR-1 being proapoptotic and miR-133 being antiapoptotic (Xu et al. 2007). Reporter assays and functional studies have shown that miR-1 targets and significantly reduces HSP60 and HSP70 protein levels, whereas miR-133 represses caspase 9 expression, thus acting as an antiapoptotic miRNA. In a recent study, miR-133b was shown to target antiapoptotic genes in prostate cancer; miR-133b is downregulated in over 75 % of prostate cancer patient samples compared with the normal tissues. Expression of miR-133b enhanced death-receptor-induced apoptosis in prostate cancer cells (Patron et al. 2012).

There are also examples of miRNAs that target prosurvival genes, leading to apoptosis. For instance, a recent report demonstrated that in HCC cells miR-449 is downregulated, leading to activation of MET mRNA, thus inhibiting apoptosis and increasing proliferation of liver cells. However, exogenous expression of miR-449 considerably reduces the growth of HCC xenograft tumors in mice (Buurman et al. 2012). Similarly, miR-497 targets insulin-like growth factor receptor type 1 (IGF1R) in colorectal cancer cells. IGF1R is implicated in colon cancer progression, and downregulation of miR-497 leads to upregulation of IGF1R, thus contributing to this malignancy (Guo et al. 2013).

Mutations in miRNAs or miRNA binding sites can affect the target gene regulation. For example, miR-146a is highly expressed in breast, ovarian, stomach, and pancreatic cancer (Shen et al. 2008; Hurst et al. 2009; B. Xu et al. 2010). A single-nucleotide polymorphism within the precursor of miR-146a predisposes individuals to papillary thyroid carcinoma (Jazdzewski et al. 2009) and can turn miR-146a antiapoptotic (Jazdzewski and de la Chapelle 2009). The key miRNA-mediated apoptotic gene networks are summarized in Fig. 19.3.

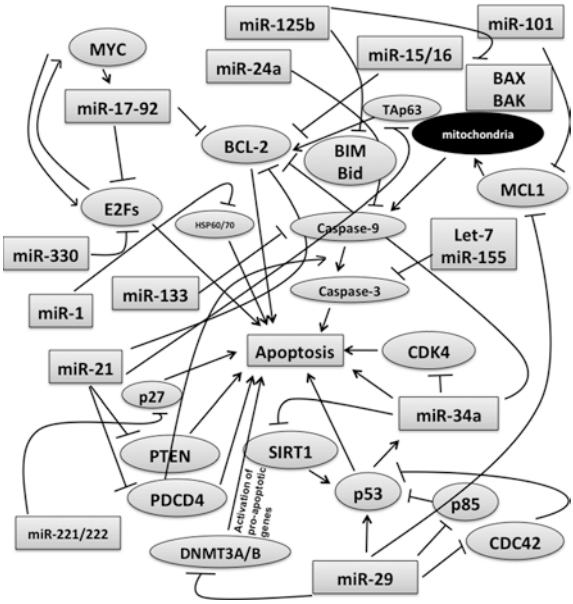

Fig. 19.3 The miRNA gene regulatory networks involved in the control of apoptosis: The activation of genes/miRNAs is indicated by *arrows*, and the targeting of mRNA by miRNA is shown by *lines ending with a block*. The diagram includes miRNAs implicated in p53, MYC, and caspase signaling pathways. (Adapted from Subramanian and Steer 2010)

19.6 Conclusions

Comprehensive understanding of gene expression patterns and protein networks in various cancer conditions has significantly increased our knowledge of the basic biology of cancer. In addition, it has also enriched our ability to develop novel diagnostic/prognostic markers as therapeutic targets. In recent years, miRNAs have emerged as major regulators of protein expression and are involved in key cellular functions, such as development, proliferation, and differentiation, and have been implicated in almost all disease conditions. Gene regulatory networks mediated by miRNAs are under intense study. Both p53 and cMYC can induce the expression of multiple miRNAs and can be regulated by sets of miRNAs that are conserved and found to be deregulated in multiple cancers (He et al. 2007a; Mendell 2008). For instance, activation of miR-17-92 mediated by cMYC is a characteristic feature in osteosarcoma and several other carcinomas. Similarly, deregulation of miR-29 is seen in sarcomas (Subramanian et al. 2010; Li et al. 2012) and other malignancies (Garzon et al. 2009; Park et al. 2009b). Further, miR-29 family members activate p53 (Park et al. 2009b) and their loss of expression can limit p53 levels, as and when required in the cell. Subsequently, loss or inactivation of p53 expression leads to downregulation of miR-34, a transcriptional target of p53 (He et al. 2007a).

Understanding the factors that stabilize cMYC and/or p53 expression in cancers can also provide valuable insights into cancer pathobiology. Although these molecular networks shed some light on the cellular functions and their role in

cancer, many questions persist, including basic ones such as whether miRNAs act as a driver or a passenger in the pathogenesis of disease. The identification and validation of mRNA targets will undoubtedly increase our understanding of the miRNA gene regulatory networks (He et al. 2007b; Ma et al. 2007; Tavazoie et al. 2008; Wang et al. 2008a). Because of the partial complementarity of miRNA to target genes, it remains a challenge to identify all targets of a given miRNA in a particular cell or tissue type. Although new molecular tools such as argonaute high-throughput sequencing of RNA isolated by cross-linking immunoprecipitation have been developed to understand the genome-wide miRNA–mRNA interactions (Chi et al. 2009), the identification of miRNA targets is complicated by mechanisms such as alternative splicing and a shortened 3′ UTR, which result in the loss of miRNA binding sites (Sandberg et al. 2008; Mayr and Bartel 2009). Further, polymorphisms in the miRNA binding sites may create or destroy miRNA binding sites (Kim and Bartel 2009). Investigating miRNA biology in cellular apoptosis can be challenging in many ways. First, a single miRNA can regulate many genes. Depending on the cell type and target availability, a miRNA can function either as an oncogene (antiapoptotic) or as a tumor suppressor (proapoptotic). For instance, miR-17-92, a predominantly antiapoptotic miRNA cluster, has also been reported to promote apoptosis in breast cancer by targeting the amplified in breast 1 gene (*AIB1*) (Hossain et al. 2006) and cyclin D1(Yu et al. 2008). Thus, the roles of miRNAs cannot be generalized across multiple cancers or cell types. Second, modulation of miRNAs can also have off-target effects. Third, deregulation in miRNA expression leads to "noise" in the cellular gene expression, and these noise levels differ in different cancers. Thus, miRNA deregulations, leading to the resultant noise in tumor cells and in surrounding stromal cells, may contribute to tumor heterogeneity and drug resistance.

MicroRNAs (miRNAs) are known to have hundreds of targets and many may function as passengers in the tumorigenic process. Driver genes are key players in tumorigenesis, and investigating the interactions between miRNAs and driver genes (driver of driver genes) will significantly enhance our understanding of tumor biology. These miRNA–gene networks can also cooperatively work with the competing endogenous RNAs (Karreth et al. 2011) and regulate or be regulated by a plethora of gene networks that are maintained in an intricate balance for normal cellular functioning. Understanding these multilayered gene regulatory mechanisms may pose a major challenge in understanding cancer biology. Focusing on key driver of driver genes may reduce the miRNA–gene network complexities to a certain extent. The future research directions for the roles of miRNAs in cancer may include (1) development of body fluid (serum/plasma) based miRNA biomarkers, (2) miRNA-based targeting of genes in a cancer signaling pathway using miRNA mimics and or ant-imiRs/sponges instead of targeting a single gene/protein product, (3) investigation of potential competing endogenous RNAs as alternative drug targets for cancer genes that are currently undruggable by regular therapeutic approaches, and (4) modulating miRNA expression levels may be explored as differentiation, intercellular communication, and stem cell based cancer therapies.

Of the 50 or more miRNAs that play a role in apoptosis (Table 19.1), it is not entirely clear how many of these miRNAs are affected by the above-mentioned factors that determine miRNA-mediated regulation of the target gene. The role of miRNAs in the control of apoptosis is already firmly established. MicroRNAs (miRNAs) have complex gene regulation mechanisms and these are determined by the presence or absence of the target genes in a given cell type. Balancing and maintaining the threshold levels of these miRNAs is another complex level of gene regulation. It is also essential to understand the molecular cross talk between miRNA and other noncoding small RNAs such as piwi-interacting RNA and repeat-associated small interfering RNA to understand the multifaceted roles of miRNAs. However, with our extensive understanding of miRNA function and gene regulation, in the future it should be possible to fine-tune and specifically control cancer-signaling genes and/or pathways using "designer" miRNAs.

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Part IV
Strategies for Cancer Treatment

Chapter 20
Markers of Cancer Stem Cells and Their Functions

Olivier Gires

Abstract Embryonic stem cells are the basis of developing organisms. They posses self-renewal capacity and pluripotency to eventually generate every cell type of the mature body. In the course of development, organs arise through morphogenesis, including the differentiation of tissue progenitor cells into mature cells. Thorough analysis of the composition and the heterogeneity of organs revealed the presence of residual tissue progenitors, which retain the capacity to repopulate organs if necessary, e.g., because of injury. Hence, organs display a hierarchical structure very comparable to the initial structures in the developing embryo, however with a loss of potency. Similarly to healthy organs, tumors have hierarchical structures comprising tumor progenitors, which give rise to the tumor bulk (i.e., more "differentiated" tumor cells). Tumor progenitors are termed cancer stem cells (CSCs) or tumor-initiating cells. In this chapter, we discuss markers commonly used to discriminate CSCs from the tumor bulk and address their functions in the context of tumor development. The main focus is on the structure and function of CD44, CD133, epithelial cell adhesion molecule, CD24, CD166, CD47, leucine-rich-repeat-containing G-protein-coupled receptor 5, and aldehyde dehydrogenase 1 in CSCs. Importantly, the option of substantial plasticity of CSCs is addressed in the light of the functions of CSC markers and their expression pattern.

Keywords Tumor-initiating cells • CD133 • CD44 • CD47 • Epithelial cell adhesion molecule

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20.1 Stem Cells, Cancer, and Cancer Stem Cells

Isolation and cultivation of healthy murine embryonic stem cells (ES cells) was first described in 1981 by Evans and Kaufman (1981). ES cells are cells originating from the inner cell mass (embryoblast) of day 4–5 healthy embryos (blastocyst). The term "embryonic stem cell" (ES cell) was coined by Martin (1981) in the same year. Normal ES cells were experimentally connected to malignant stem cells, the description of ES cells actually being preceded by work on so-called embryonic carcinoma cells (EC cells). EC cells derived from teratocarcinomas and, although displaying properties of genuine ES cells, were flawed by various genetics abnormalities also seen in the germ cell tumors from which they were isolated (Martin and Evans 1974). Hence, a connection between normal and malignant stem cells dates back to the initial descriptions of ES cell types, and EC cells can be seen as their first described malignant counterparts (Andrews et al. 2005). In the past two decades, there has been strong focus on the study of cancer stem cells (CSCs; also termed tumor-initiating cells) of leukemia and solid tumors of various origins. This led to the view that cancers, similarly to healthy organs, display a hierarchical structure, with CSCs as the origin of malignancies (Visvader and Lindeman 2008). However, as will be discussed later, the theory of CSCs is being debated and clearly has proponents and opponents (Azvolinsky 2012).

The concept of CSCs has its roots in the fact that tumor cells differ in their oncogenic potential in vivo in animal models and on retransplantation in humans. In 1960, Southam and Brunschwig (1960) reported their work on the autotransplantation of human cancer in recognition of the comparatively low frequency of metastasis formation even in the presence of substantial numbers of circulating tumor cells in patients. Their goal was "to probe for possible explanations of the presumed failure of many disseminating cancer cells to implant and grow." In fact, only five of 27 autotransplantations gave rise to tumor nodules, and a minimum of 1,000,000 tumor cells were required for the outgrowth of tumor nodules on subcutaneous autotransplantation. Two of five patients developed nodules only after inoculation of 100×10^6 cells. The very same issues and concerns about the quality and nature of transplanted cells seen nowadays were raised in the discussion of this particular publication. The authors took into account that cell counting can be error-prone, that the viability of cells might be affected, and that nontumor cells mix in the inoculated samples. However, even after tentative correction of the numbers of transplanted tumor cells, a minimum of 10,000 cells appeared necessary to induce malignant growth in vivo (Southam and Brunschwig 1960).

Today′s view of the hierarchical structure of tumors is that a subpopulation of malignant cells with self-renewal capacity and the potential to aberrantly differentiate into the bulk of tumor cells initiate cancers (Visvader and Lindeman 2008). Accordingly, tumors are organized in a hierarchical manner very comparable to normal tissue, however with a loss of control of homeostasis of the progenitor/

stem compartment. The gold standard for the characterization of CSC relies on serial xenotransplantations of sorted human tumor cells into immunocompromised mice. The first report of sorted tumor cells with differential oncogenic properties addressed CSCs of acute myeloid leukemia (AML) and demonstrated that AML contained an average of one tumor-initiating unit per 250,000 cells and that CSCs displayed a $CD34^{+}/CD38^{-}$ phenotype (Lapidot et al. 1994). The first demonstration of the existence of CSCs in solid tumors was almost 10 years later with the finding of $CD44^{+}/CD24^{low/-}$/lineage^{-} breast cancer stem cells (Al-Hajj et al. 2003). Starting from this seminal work, sorting of CSCs from numerous solid tumors was achieved with cellular antigens such as CD44, CD133, epithelial cell adhesion molecule (EpCAM), CD24, CD166, CD47, leucine-rich-repeat-containing G-protein-coupled receptor 5 (Lgr5), and aldehyde dehydrogenase (ALDH) 1 alone or in combination, and after negative selection of lineage-negative cells to deplete immune cells (Visvader and Lindeman 2008).

Now a decade later, some features of markers of CSCs are becoming more evident or even emerging, and potentially shed further light on the biology of these cells. Generally speaking, marker-positive cells can be serially transplanted and give rise to new tumors with a cellular composition reminiscent of the primary malignancy. However, CSCs from the same tumor entity can be characterized by differing sets of markers. For example, colon cancer stem cells were described as the $CD133^{+}$ subpopulation (O'Brien et al. 2007; Ricci-Vitiani et al. 2007) or as the $CD44^{+}/EpCAM^{high}$ subpopulation (Dalerba et al. 2007). The same CSC markers are commonly found in numerous cancers of totally different localization. Markers commonly used for the enrichment of CSCs could be expressed as a result of transformation or, alternatively, impact on the oncogenic potential of CSCs and be seen as causative. Besides the continuing debate on the applicability of the CSC theory to all tumor entities or even to all patients with a given cancer type (Magee et al. 2012), a high degree of plasticity is reckoned in CSC biology, where cells may transdifferentiate from a more proliferative to a more migratory CSC state (Biddle et al. 2011). The first differences of marker expression between these CSC populations have been reported recently (Biddle et al. 2011). Therefore, current work focuses on the study of the function of CSC markers in the regulation of the phenotype and the aptitudes of these cells.

The following sections will delineate present knowledge on the function of CSC markers.

20.2 Characteristics and Prerequisites for Tumor-Initiating Cells

With the emergence of the CSC theory, some accepted notions in carcinogenesis appeared to be challenged or even refuted. Soon the CSC theory was in opposition to the multistep carcinogenesis theory, which claims that normal cells accumulate successive mutations in a time-dependent manner and transform into oncogenic

clones. The cellular functions affected by these "driver mutations" have been compiled in one of the most frequently cited articles, "The hallmarks of cancer" by Hanahan and Weinberg (2000), and refer to:

- Insensitivity to growth inhibitors
- Self-sufficiency in growth signals
- Limitless replication
- Sustained angiogenesis
- Evasion of apoptosis
- Tissue invasion and metastases

The greatest discrepancy between both theories concerned the tumorigenic potential of individual cells of a formed tumor. According to the multistep carcinogenesis theory, each cell within a tumor is a clone of the founding cell and, in principle, harbors comparable tumorigenic potential. In the case of a CSC-driven tumor, cells strongly differ in their capacity to form novel tumors, with CSCs being the only oncogenic cells, whereas more "differentiated" tumor cells of the bulk remain silent in this respect. This discrepancy is not only of academic relevance but is supposed to have a great impact on cancer treatment. If only a subset of cells are tumorigenic, treatment modalities must be adapted in order to target CSCs in addition to or instead of the tumor bulk in order to avoid recurrence and high lethality.

The initial debate and strict secession of both theories with all their consequences were slackened and both are subject to steady revision. Obviously, the above-mentioned hallmarks of cancer apply as requirements to CSCs too. Further, mutations or epigenetic changes in cells of a clone might generate cells with differential potential to initiate novel tumors, and external influences from the tumor surroundings must be considered. Thus, the consensus might be that indeed several mutations are required in order to transform cells (multistep carcinogenesis), resulting in tumor-initiating cells (CSC theory), which can partly lose their ability to induce tumors owing to transdifferentiation and loss of support from the surrounding tissue. A simplified bottom line is that tumorigenesis cannot be perceived as a static process but must rather be seen as a dynamic act, which implies substantial plasticity in tumor (tumor-initiating) cells.

What remains more than ever is the urgent need for broad knowledge of the prerequisites, which determine the phenotype of tumor initiators of solid tumors, since this knowledge is a prerequisite for the refinement of cancer therapies. Depending on the actual localization in the body and on the stage in the process of carcinogenesis, CSCs most probably have differing requirements. Cells which transform and proliferate within an organ to form a local tumor are different from disseminating/circulating tumor cells, which are themselves different from cells of micrometastasis and metastasis. In other words, the abilities cells require in order to "perform" in their malignant developmental state are different, suggesting that cells adapt (plasticity). Accordingly, two major levels of prerequisites can be defined, which affect (1) regulators of limitless replication, invasive growth at the site of transformation, evasion of apoptosis, and sustained angiogenesis and (2) regulators of migration, invasion, engraftment, and communication. The first

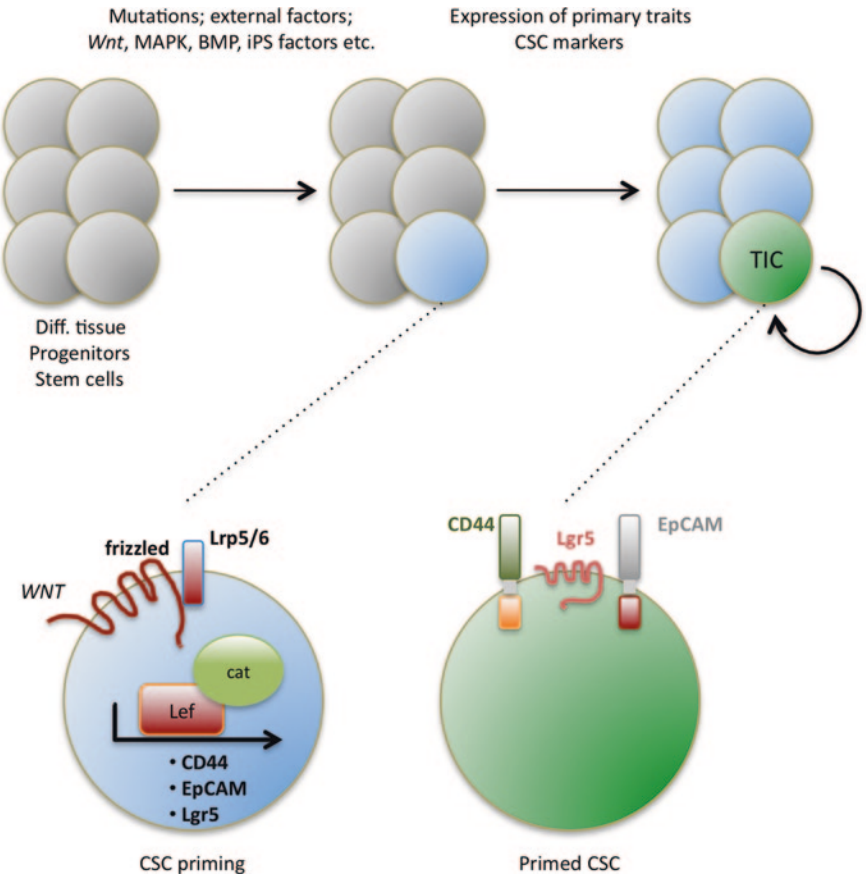

Fig. 20.1 Priming and regulation of cancer stem cells (*CSC*). Differentiated cells, progenitors and/or stem cells require mutations and/or the activity of external factors to prime them to become CSCs. Among these stimuli are Wnt, mitogen-activated protein kinase (*MAPK*), and bone morphogenetic protein (*BMP*) signaling, and expression of induced pluripotent stem cell (*iPS*) factors. On induction of these signals, cells de novo express or overexpress primary trait CSC markers such as CD133, CD44 and epithelial cell adhesion molecule (*EpCAM*) and thus become primed. *Lgr5* leucine-rich-repeat-containing G-protein-coupled receptor 5, *Lrp* lipoprotein-receptor-related protein, *TIC* tumor-initiating cell

regulators are mandatory for cells to become CSCs within the organ from which they originate and hence will be termed primary traits in the following (Fig. 20.1), whereas the second regulators are more confined to secondary events in carcinogenesis, which are associated with invasive growth and metastasis formation (Fig. 20.2). In this case, regulators need not necessarily to be single proteins but might also represent entire pathways or combinations thereof.

Priming of CSCs from differentiated cells, progenitors, or stem cells will rely on the induction of primary traits and result in CSCs with various capacities, including the formation of large, space-occupying tumors or locoregional/distant metastases

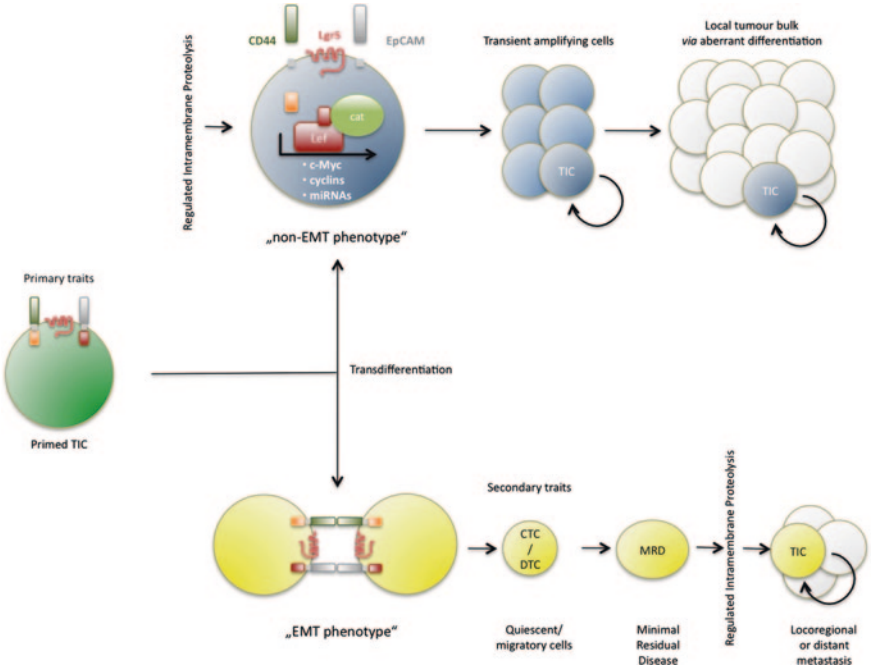

Fig. 20.2 Different fates of primed CSCs. Primed CSCs express various markers of primary traits, which can be regulated at the functional level on regulated intramembrane proteolysis (RIP). On RIP, cells become activated to proliferate and differentiate to a tumor bulk in which CSCs will represent a minor fraction of the cells. Under conditions of low RIP activity and/or lack of inducing ligands, cells can adopt a communicative and rather quiescent phenotype. Such cells can acquire additional (secondary) trait markers and advance to circulating tumor cells (*CTC*) or disseminated tumor cells (*DTC*) and be cells of the minimal residual disease (*MRD*). After engraftment, reactivation of proliferation, for example, on RIP and induction of primary traits markers, will lead to the formation of secondary tumors at distant sites or metastases. *EMT* epithelial–mesenchymal transition, *miRNAs* microRNAs

(Fig. 20.2). Depending on the cell type affected by this transformation, the need for changes in the cell program will be different and will increase from a tissue stem cell to a fully differentiated mature cell. Although experimental evidence remains scarce, malignant reprogramming is presumably more efficient in tissue stem cells than in differentiated cells. Although common sense is that a certain tumor size is required for metastatic spread, this is differs across entities, and very early spread can be observed with small tumors too. Hence, the acquisition of secondary traits, which govern the metastatic potential of tumor initiators, could occur at early or more advanced stages of carcinogenesis (Fig. 20.2). Accordingly, CSCs and their progeny must be seen as smooth transitions of various states of tumor cells, which all ultimately rely on regulation of gene expression and function.

Obviously, a cell will not receive signals incorporating all aspects of primary or secondary traits from a single molecule or after a singular mutation of the genome.

It is rather to be expected that orchestrated functions of various proteins result in the variable phenotype of CSCs, fine-tuning of their capacity, and regulation of plasticity depending on the actual needs. In the following, reported CSC markers will be reviewed and classified according to their molecular functions and potential role in the regulation of CSC phenotypes. Unless mentioned otherwise, the markers discussed have been defined to be bona fide CSC-associated proteins on serial transplantations of human marker-positive tumor cells into immunodeficient mouse strains.

20.3 CD133

Prominin-1 is an approximately 120-kDa glycoprotein, which is also termed CD133 according to the cluster of differentiation classification, and was first identified as a marker of hematopoietic stem cells (Yin et al. 1997) and subsequently cloned (Miraglia et al. 1997). CD133 is composed of an N-terminal extracellular domain followed by two large extracellular loops, which are strongly N-glycosylated, and an intracellular C-terminus (Corbeil et al. 2001). Judging and evaluating the potential contribution of CD133 to the phenotype of CSC might be a demanding task, since the actual function of this molecule is only partly understood. The antigens AC133 and AC141, which most probably represent hyperglycosylated versions of CD133 and are recognized by the two major antibodies used for detection of CD133 are abundantly expressed in stem cells (Yin et al. 1997) such as embryonic epithelium (Marzesco et al. 2005), brain stem cells (Uchida et al. 2000), and hematopoietic stem cells (Freund et al. 2006), and in cancers such as leukemias (Horn et al. 1999) and retinoblastomas (Maw et al. 2000). AC133/AC141 antibodies were very useful to isolate CSCs from colon (O'Brien et al. 2007; Ricci-Vitiani et al. 2007), pancreas (Hermann et al. 2007), gallbladder (Shi et al. 2010), ovarian (Curley et al. 2009), lung (Bertolini et al. 2009), and brain (Singh et al. 2004) cancers, childhood malignant melanoma (Al Dhaybi et al. 2010), and Ewing′s sarcoma (Suva et al. 2009). Especially with the report of a seemingly strict separation of CD133 expression in CSCs but not in non-CSCs in colon cancer (O'Brien et al. 2007; Ricci-Vitiani et al. 2007), the notion emerged that the CD133 protein is exclusively expressed in the CSC subpopulation in cancers. This notion was vigorously challenged when Shmelkov et al. (2008) reported that $CD133^+$ and $CD133^-$ cells have tumor-seeding capacity in metastatic colon cancers and that CD133 is ubiquitously expressed in murine and human differentiated colonic epithelium, questioning the validity of CD133 as a marker for CSCs at least in this particular entity (Shmelkov et al. 2008). In line with this report, a $CD133^-$ cancer cell line generated from colon carcinoma retained tumor-initiating and aberrant differentiation potential (Navarro-Alvarez et al. 2010). In glioblastomas, although initial reports focused on $CD133^+$ CSCs, more recent data support the existence of $CD133^-$ cells with CSC properties too (Beier and Beier 2011). One possible explanation for this obvious discrepancy is that AC133-specific and AC141-specific antibodies do not react with the hypoglycosylated CD133 protein

present in more differentiated tumor (stem) cells anymore (Miraglia et al. 1997; Corbeil et al. 2000; Kemper et al. 2010), and hence mimic a loss of CD133 antigen. Additionally, a dependency on conformation for antibody binding was discussed (Miraglia et al. 1997). Thus, a superior expression in stem cells and CSCs only holds true for the AC133/AC141 antigen, whereas a hypoglycosylated and/or conformational variant of CD133 remains expressed in more differentiated tumor cells (Kemper et al. 2010). More precisely, a genetic isoform of CD133 termed AC133-2, which lacks exon 4, resulting in a deletion of nine amino acids in the N-terminus of the protein, is the major CD133 variant in hematopoietic stem cells (Yu et al. 2002). In other words, AC133/AC141 and CD133 are not similar since only a subfraction of $CD133^+$ cells actually display the AC133/AC141 epitope (Fargeas et al. 2011). These issues and the repercussion on CSC isolation and biology with respect to CD133 were discussed in depth by Bidlingmaier et al. (2008).

The evaluation of the contribution and function of CD133 to the biology of CSCs is further hindered by the existence of several splice variants (Fargeas et al. 2007), the respective impact of which on the function of CD133 is not clear. Nonetheless, in hematopoietic stem cells, CD133 expression correlates with a more pronounced multipotent phenotype than in $CD34^+/CD133^-$ cells (Bauer et al. 2008). During asymmetric division of hematopoietic stem cells, CD133 is primarily distributed to daughter cells with a stem cell phenotype (Freund et al. 2006), as is the case for neuroepithelial cells. Furthermore, budding of CD133-containing membranous particles termed prominosomes has been reported during differentiation of neuroepithelia to radial glial cells and neuron-generating progenitors (Marzesco et al. 2005). Release of $CD133^+$ particles from the apical membrane of neuroepithelia might be a means of intercellular communication and/or disposal of proteins that regulate stemness.

Studies related to the function of CD133 addressed the effects of a knockdown in cancer cells. In colon carcinoma cells, in which CD133 is a reported CSC marker with prognostic significance, the repression of CD133 expression had no effect whatsoever on proliferation, colony formation, migration, and invasion (Horst et al. 2009). Thus, CD133 apparently lacks a function in colon carcinogenesis (Horst et al. 2009). In the murine model, CD133 expression is characteristic of intestinal cells, which, on hyperactivation of the Wnt signaling pathway, are prone to transformation and will populate the intestine with neoplastic cells (Zhu et al. 2009). Accordingly, stable expression of a glycosylated form of CD133 in otherwise $CD133^-$ cells was associated with a 1,000-fold increase in tumor-seeding capacity (Canis et al. 2013). Recent studies finally concentrated on the molecular function(s) of CD133 and its interplay with major signaling pathways. Takenobu et al. (2011) demonstrated a role for CD133 in the promotion of proliferation, anchorage-independent growth, and tumor formation in vivo in neuroblastoma cells. Concomitantly, CD133 suppressed neuroblastoma cell differentiation via the inhibition of the transcription of RET tyrosine kinase depending on the phosphatidylinositol 3-kinase pathway. In head and neck squamous cell carcinomas, CD133 expression was induced on enrichment of CSCs, and CD133 enhanced tumor initiation, expression of stemness genes, and self-renewal

capacity in these cells (Chen et al. 2011b). The observed phenotype was accompanied by a substantial increase in the phosphorylation and, hence, in the activation status of Src kinase (Chen et al. 2011b). Thus, evidence is increasing that CD133 is not only a marker for CSCs but is also most probably a driver of the phenotype of these cells and a *primary trait regulator*.

The relevance to CSCs as a primary trait regulator is as follows:

- Pluripotency and differentiation capacity (asymmetric division, plasticity)
- Communication with microenvironment
- Migratory capacity and engraftment (metastasizing CSCs)
- Suppression of differentiation via RET tyrosine kinase
- Regulation of tumor-initiating potential and epithelial–mesenchymal transition (EMT)

20.4 CD44

Proteins encoded by the CD44 gene constitute a large family of at least 20 variants owing to differential splicing and posttranslational modifications. Therefore, only global effects of CD44 proteins can be discussed here, without going into the differentiated function of each CD44 isoform in detail. Structurally, CD44 is a single transmembrane protein with a comparably short intracellular domain (72 amino acids), whose expression is regulated by the Wnt signaling pathway via β-catenin (Wielenga et al. 1999), as is the case for other CSC markers such as EpCAM and Lgr5 (Fig. 20.1). CD44s is the standard isoform of CD44, which incorporates exons 1–5 and 16–20 of the gene, whereas variable isoforms are generated on the incorporation of additional exons via differential splicing. CD44s was used to enrich CSCs from colon carcinomas (Dalerba et al. 2007), head and neck carcinomas (Prince et al. 2007), non-small-cell lung cancer (Leung et al. 2010), hepatocellular carcinoma (Zhu et al. 2010), and breast cancer (Al-Hajj et al. 2003). It is striking that CD44, together with CD133 and EpCAM, is one of the most frequently used markers for the enrichment of CSCs from primary tumors of totally different localizations (Lobo et al. 2007), raising the question of whether it fulfills essential tasks in CSCs especially in light of its partially high and ubiquitous expression in tumors (for a review, see Zoller 2011)). As for EpCAM, it is the further overexpression of CD44 rather than a de novo expression which appears characteristic of CSCs. It is, however, fair to mention that this notion is being debated, for example, for the case of CSCs from head and neck squamous cell carcinomas. The actual expression patterns of CD44 in CSCs in head and neck squamous cell carcinomas remain a matter of debate. Initial publications reported a subpopulation of head and neck squamous cell carcinomas of less than 10 % of the tumor bulk on average, which displayed tumor-initiating potential when xenotransplanted into immunodeficient mice (Prince et al. 2007). However, these findings contradict the almost ubiquitous expression of CD44s and the alternative splice variant

CD44v6 in head and neck squamous cell carcinomas and in most normal epithelia of the head and neck area (Kawano et al. 2004; Mack and Gires 2008). However, be it the de novo expression or the further increase of expression in cells with tumor-initiation potential, CD44 appears to be a central protein in the characterization and possibly in the regulation of the CSC phenotype.

CD44 is the major hyaluronan receptor and as such induces adhesion to and communication with extracellular matrix of the microenvironment. Its second function is the transmission of signals from the plasma membrane to the nucleus on regulated intramembrane proteolysis (RIP) (Ponta et al. 2003). Activation of the signaling pathway of CD44 relies on RIP to deliver the intracellular domain of CD44 into the nucleus (Figs. 20.1, 20.2). On nuclear translocation, the intracellular domain of of CD44 binds to 12-*O*-tetradecanoylphorbol-13-acetate-responsive elements within promoters to induce transcription of target genes, including CD44 itself (Nagano and Saya 2004) and is involved in cell transformation of rat fibroblasts (Pelletier et al. 2006). Functionally, CD44 plays a role in the balance of survival, apoptosis, and chemoresistance in colorectal cancers as shown in the $Apc^{min/+}$ mouse model, whereas it did not impact proliferation of cells (Zeilstra et al. 2008). Activation of CD44 on interaction with hyaluronan results in induction of the p300 acetyltransferase, acetylation of β-catenin and nuclear factor κB , and eventually in upregulation of genes involved in multidrug resistance and protection from apoptosis (Bourguignon et al. 2009b). Furthermore, hyaluronan-dependent activation of CD44 induces protein kniase Cε and Src kinase, which are required for activation of the phosphorylation of Nanog and Twist, respectively. Nanog and Twist are transcription factors that are involved in the regulation of the microRNAs miR-21 and miR-10b, themselves regulators of tumor suppressors and GTPases, which are active in cytoskeleton reorganization during EMT and metastasis formation, and of inhibitor of apoptosis and multidrug-resistant protein 1 (Bourguignon et al. 2010). Theferore, CD44 has the potential to regulate the number and function of vital CSCs via the regulation of apoptosis and chemoresistance. Consequently, CD44 expression might impact on the pool of CSCs in vivo, and can be ranked as a *primary trait regulator*.

The relevance to CSCs as a primary trait regulator is as follows:

- Inhibition of apoptosis and induction of chemoresistance
- Migratory capacity and engraftment
- Autonomy from tissue integrity (disseminated/circulating CSCs)
- Activation of Nanog (stemness) and Twist (EMT)
- Related to Wnt, protein kinase C, Src kinase, and nuclear factor κB

20.5 EpCAM

EpCAM was first described in 1979 and as such is one of the longest known tumor antigens (Herlyn et al. 1979). The history of EpCAM in oncology is not only long-lasting but also controversial and twisted. Owing to its strong expression in tumor

tissue, EpCAM served early on as a target for therapeutic monoclonal antibodies (Riethmüller et al. 1994, 1998). At the time of clinical development of therapeutic antibodies such as edrecolomab, EpCAM was perceived as a cell adhesion molecule (Litvinov et al. 1994a, b) involved in the modulation of cell adhesion mediated by classical cadherins (Litvinov et al. 1997) and associated with proliferation and loss of differentiation (Litvinov et al. 1996). Finally and after evaluation of thousands of patients, conclusive studies revealed a lack of efficacy of edrecolomab (Fields et al. 2009), a matter which was intensely discussed (Schmoll and Arnold 2009; Gires and Baeuerle 2010). With the discovery of a frequent and strong expression of EpCAM in CSCs, another dimension was added, which revived interest in this molecule (Gires et al. 2009).

So how can all these controversial views of EpCAM be brought together? Possibly through the finding that EpCAM is, as many other cell adhesion molecules, a dual-function protein, which is involved not only in cell adhesion processes but also in signaling and cell cycle regulation. Selective activation of differential functions of EpCAM and impacts of therapeutic antibodies on these functions might account for the numerous facets of this molecule.

Structurally, EpCAM is an integral transmembrane protein composed of a large extracellular domain, one transmembrane region, and a small intracellular domain of 26 amino acids (Chong and Speicher 2001). The extracellular domain of human EpCAM is N-glycosylated at three distinct asparagine residues (Asn-76, Asn-111, and Asn-198) (Chong and Speicher 2001). Glycosylation patterns have proven to be differential in normal tissue versus tumor, with a hyperglycosylation being characteristic of the malignant state (Pauli et al. 2003). This finding is of significance since N-glycosylation of Asn-198 is associated with enhanced stability and retention of EpCAM at the cell surface (Munz et al. 2008). Although EpCAM is mostly glycosylated in tumors, a systematic approach for the analysis of an effect of glycosylation of EpCAM on the stability in vivo is lacking up to now.

Already in the first work regarding the isolation of CSCs from solid tumors in breast carcinomas, EpCAM (termed ESA therein) allowed an approximately tenfold enrichment of CSCs as compared with the initial signature of $CD44^+/CD24^-/lineage^-$ breast cancer stem cells (Al-Hajj et al. 2003). Later, EpCAM was described as part of the CSC signature in colorectal carcinoma (Dalerba et al. 2007), hepatocellular carcinoma (Terris et al. 2010; Yamashita et al. 2009), extrahepatic cholangiocarcinomas (Wang et al. 2011), ovarian carcinoma (Meirelles et al. 2012), K-ras(G12D)-induced lung adenocarcinoma (Cho et al. 2011), hepatoblastoma (Ruck et al. 2000), and pancreatic carcinomas (Li et al. 2007). As for CD133, EpCAM overexpression is not only associated with malignant stem cells. The temporal and cell-type-specific regulation of EpCAM expression is well documented in the morphogenesis, injury, and transformation of liver cells (de Boer et al. 1999). Liver progenitors express EpCAM to high levels and downregulate this expression throughout differentiation until no residual EpCAM can be detected in terminally differentiated hepatocytes (de Boer et al. 1999; Gires 2011; Yoon et al. 2011). Cells driving the processes of liver repopulation after injury or malignant transformation typically reexpress EpCAM (de Boer et al. 1999; Ruck et al. 2000; Yamashita et al. 2009).

Two features of EpCAM most probably led to its poor consideration as a possible "driver" of CSCs: (1) EpCAM was for a long time perceived as a cell adhesion molecule without transforming potential and (2) EpCAM is per se expressed in most carcinomas, as is CD44, and does, as a single marker, not allow the discrimination of CSCs versus non-CSCs. In fact, it is the even stronger expression of EpCAM (up to tenfold) in CSCs which distinguishes it from the bulk of tumor cells. However, over the past decade evidence has accumulated showing that EpCAM is an oncogenic signaling receptor. Expression of EpCAM in otherwise negative cells results in increased proliferation, cell cycle progression, and induction of the proto-oncogene c-Myc (Munz et al. 2004), whereas reduction of EpCAM expression in breast carcinoma cell lines mediated by small interfering RNA induced a loss of proliferation and invasion capacity (Osta et al. 2004). Targeted disruption of the *Epcam* gene in mice is embryonically lethal, which underscores the essential roles in regular development and morphoregulation in vivo (Nagao et al. 2009). One explanation for the apparent essential role of EpCAM comes from very recent findings which revealed a direct effect of EpCAM on the regulation of the cell cycle. EpCAM regulated the transcript and protein levels of cyclin D1, which is instrumental in the G1 phase during cell cycle progression (Chaves-Pérez et al. 2013). Following the report on EpCAM′s function in the regulation of c-Myc and proliferation (Munz et al. 2004) great efforts were made by various laboratories to elucidate EpCAM′s signaling pathway at the molecular level. RIP, as described for CD44, Notch, L1, and CD166, can be seen as a common means to switch between adhesive and signaling properties of EpCAM (Fig. 20.2). Human EpCAM is firstly cleaved by ADAM proteases (ADAM17) to generate a so-called C-terminal fragment (CTF) and to release the ectodomain termed EpEX. CTF-EpCAM is then a substrate for the γ-secretase complex, which cleaves CTF-EpCAM in the transmembrane domain, yielding an intracellular domain termed EpICD (Maetzel et al. 2009). EpICD is the signaling moiety of EpCAM, which translocates to the nucleus and, together with components of the Wnt signaling pathway (i.e., β-catenin, FHL2, and LEF) induces the transcription of target genes (Maetzel et al. 2009).

In addition to the expression levels of CSC markers themselves, regulation of their function in the shaping of CSCs can occur at a second level. Expression and activity of essential components of RIP will impact on selected CSC marker functions and distinguish between a "signaling phenotype" and a "communicative phenotype" after cells have been primed to CSCs (Fig. 20.2). For the case of EpCAM, proteases required for proteolytic activation (i.e., TACE and presenilin) and the interaction partner FHL2 are substantially upregulated in various malignant tissues (Johannessen et al. 2006; Kenny 2007; Selkoe and Wolfe 2007; Wang et al. 2007; Li et al. 2008; Merchant et al. 2008), and might hence explain the strong differences in EpCAM activation observed in colon carcinoma as compared with normal colonic mucosa (Maetzel et al. 2009).

Components of the Wnt pathway crucially participate in the maintenance of the stem cell phenotype and are involved in various malignant situations (Reya et al. 2003; Reya and Clevers 2005), The *Epcam* gene itself is under the control of Tcf4

(Yamashita et al. 2007), suggesting feedback between EpCAM expression and EpCAM signaling. Cyclin D1 and c-Myc are early transcriptional target genes of both Wnt (He et al. 1998; Shtutman et al. 1999) and EpCAM signaling (Chaves-Pérez et al. 2013; Munz et al. 2004), and c-Myc is one of the essential switches of the transcriptional programs from adult stem cells to ES cells (Wong et al. 2008). Further, c-Myc was sufficient to induce an ES-cell-like transcriptional signature in normal and cancer cells and its expression enhanced the proportion of CSCs in human keratinocytes transformed by Ras and IκBα 150-fold (Wong et al. 2008). Together with the fact that EpCAM is highly expressed and an essential factor in the maintenance of stemness in murine and human ES cells (Gonzalez et al. 2009; Ng et al. 2010), possibly through its ability to regulate the promoters of the reprogramming genes Oct4, c-Myc, Sox2, Nanog, and KLF4 (Lu et al. 2010), these data strongly suggest a regulatory function in CSCs. This hypothesis is further supported by the recent report of functional subpopulations of CSC which differ morphologically and according to their cell surface markers. Biddle et al. (2011) reported the existence of two subpopulations of CSCs in carcinomas with an epithelial and proliferative phenotype (non-EMT CSC) and with a mesenchymal and migratory phenotype. The non-EMT CSCs were $CD44^{+}/EpCAM^{high}$, whereas the EMT CSC were $CD44^{+}/EpCAM^{low}$. Both CSC types were able to generate tumors in vivo, however with a decreased growth rate for EMT CSCs compared with non-EMT CSCs (Biddle et al. 2011). This was also reflected in the transdifferentiation potential: 100 % of non-EMT CSCs were bipotent and generated both subpopulations in vivo, whereas only 50 % of EMT CSCs were bipotent. This difference was even more pronounced in lymph node metastases, where only 29 % of EMT CSCs retained a bipotent phenotype. In contrast, only EMT CSCs disseminated to lymph nodes, demonstrating their invasive potential (Biddle et al. 2011).

In summary, $EpCAM^{high}$ emerged as an important primary trait regulator of proliferation and pluripotency via its capacity to signal in combination with members of the Wnt pathway. EpCAM appears very comparable to CD44, as it is frequently overexpressed in CSCs, alone does not allow the differentiation of CSCs and non-CSCs, and is as a central regulator of various aspects of the CSC phenotype.

The relevance to CSCs as a primary trait regulator is as follows:

- Self-renewal and differentiation potential
- Regulator of non-EMT CSCs versus EMT CSCs
- Switch between quiescence and proliferation
- Related to Wnt and phosphatidylinositol 3-kinase signaling
- Communication with microenvironment

20.6 CD24

CD24 is a small, glycosylated adhesion molecule, which was first described in normal B and T cells (Kay et al. 1991). It is a mucin-type protein, which is anchored in the plasma membrane via glycosylphosphatidylinositol and interacts

with P-selectin. A high degree of variability of glycosylation of CD24 generates an uncommon heterogeneity with CD24 proteins ranging from 20 to 70 kDa depending on the tissue and cell type of origin. Heterogeneity is also reported in the functions of CD24. It is implicated in T cell co-stimulation, regulation of homeostatic proliferation of dendritic and T cells, growth and metastasis of cancer cells, and apoptosis (for a review, see Liu and Zheng 2007). In accordance with the substantial differences in glycosylation, CD24 molecules have numerous different ligands, which strongly differ with the organ in which CD24 is expressed. Natural ligands are P-selectin, CD24 itself, fibronectin, and the L1 receptor (Liu and Zheng 2007).

The picture of an association of CD24 with the signature of CSCs is almost as heterogeneous as its reported functions. For the case of breast-cancer-derived CSCs, the lack or low expression of CD24 along with high expression of CD44/EpCAM is characteristic (Al-Hajj et al. 2003). In contrast, pancreatic cancer stem cells have been defined as $CD44^+/CD24^+/EpCAM^+$ cells (Li et al. 2007). As for EpCAM, transdifferentiation associated with changing levels of CD24 has been reported with the generation of $CD44^+/CD24^{-/low}$ cells with CSC capacities from nontumorigenic mammary $CD44^{low}/CD24^+$ cells after expression of oncogenic Ras in human mammary epithelial cells additionally overexpressing human TERT and SV40 large and small T antigen, and similarly in MCF10A breast carcinoma cells (Morel et al. 2008). It was demonstrated that single $CD24^+$ cells have the ability to transdifferentiate into heterogeneous $CD24^+/CD24^-$ and homogeneous $CD24^-$ populations, the latter having features of CSCs in vitro (formation of mammospheres) and in vivo (tumor generation) (Morel et al. 2008). Interestingly, these cells also underwent changes attributed to the process of EMT, and transforming growth factor β, an inducer of EMT, sped up the appearance of $CD24^-$ cells from $CD24^+$ precursors. In line with these findings, the absence of CD24 in breast cancer stem cells correlated with invasion. Oppositely, conditional expression of CD24 in mammary carcinoma cell lines resulted in an enhancement of tumorigenic and metastatic potentials of the cells (Baumann et al. 2005). CD24 expression was associated with increased proliferation, adherence to fibronectin, spreading, and migration. These controversial results are hardly reconcilable. However, if $CD24^+$ cells represent a pool of precursors of $CD44^+/CD24^{-/low}$ CSCs (Morel et al. 2008), ectopic expression of CD24 might trigger differentiation/signaling pathways which yield potent CSCs even in the presence of CD24 and therefore increase tumor formation. Transdifferentiation of non-CSCs to CSCs or even of non-EMT CSCs to EMT CSCs, as monitored on their expression of CD24, further underscores the notion that CSCs display great plasticity. Owing to these strongly controversial findings, classification of CD24 as a primary or secondary trait regulator is barely practicable.

The relevance to CSCs as a primary/secondary trait regulator is as follows:

- Switch between quiescence and proliferation
- Pluripotency and differentiation capacity (plasticity)
- Migratory capacity and engraftment (metastasizing CSCs)

20.7 ALDH

Human ALDHs compose a family of at least 19 genes subdivided in 11 families and four subfamilies (Ma and Allan 2010). These $NAD(P)^+$-dependent enzymes catalyze the oxidation of aldehydes to carboxylic acids and are essential owing to the cytotoxic and carcinogenic features of aldehydes. Assessment of ALDH1 enzymatic activity is mainly performed using the Aldefluor® assay. $ALDH1^{high}$ is, in conjunction with the expression of the catalytic subunit of telomerase and the ATP-binding cassette membrane transporter ABCG2, characteristic of normal stem cells of most tissues investigated. $ALDH1^{high}$ is also a marker for CSCs of malignancies of the breast, lung, head and neck, pancreas, cervix, prostate, liver, colon, and bladder (reviewed in Ma and Allan 2010). Central cellular processes such as differentiation, proliferation, morphoregulation, and development are directly and/or indirectly regulated by the enzymatic activity of ALDH1 family members. For example, ALDH1 members catalyze the final and irreversible step in the conversion of retinol to retinoic acid, regulating the production of retinoic acid as a central molecule involved in gene transcription and in (stem) cell differentiation (Gudas and Wagner 2011). ALDH1 enzymes confer cytoprotective effects and resistance to alkylating chemotherapeutic drugs such as cyclophosphamide via direct recycling of aldehydes (Ma and Allan 2010) and indirect pathways involving the secretion of cytokines. Similarly to CD133 and very much in accordance with its role in detoxification, ALDH1 is upregulated as a result of cellular stress (Zhang et al. 2010), which will be encountered, for example, in more central areas of tumors or in the circulation during dissemination. A subset of cells from patients with colitis with an $ALDH^+/EpCAM^+$ phenotype is the origin for the transition from a chronic ulcerative colitis to an overt colorectal cancer (Carpentino et al. 2009). Thus, $ALDH^+/EpCAM^+$ potentially marks premalignant CSCs in a situation associated with higher predisposition to develop certain cancer types. ALDH can be classified as a primary and secondary trait regulator.

The relevance to CSCs as a primary/secondary trait regulator is as follows:

- Switch between quiescence and proliferation
- Low apoptosis and high chemoresistance
- Regulation of differentiation

20.8 Lgr5

Lgr5 is a seven-span transmembrane protein whose expression is regulated by the Wnt signaling pathway (Van der Flier et al. 2007). In line with the long-known implication of Wnt signaling in various stem cells, including ES cells, hematopoietic stem cells, colonic crypt stem cells, hair follicle progenitors, and other adult stem/progenitor cells, Lgr5 efficiently marks most of these stem cell types (Haegebarth and Clevers 2009). Lgr5 is expressed in adult intestinal stem cells at

the crypt bottom, in crypt base columnar cells, which are small cycling cells which represent intestinal stem cells (Barker et al. 2007). Using elaborate transgenic animal models including markers to track Lgr5$^+$ cells and their progeny, Barker et al. (2007) demonstrated that all differentiated cell types in the intestinal epithelium, including Paneth cells, enterocytes, goblet cells, tuft cells, and enteroendocrine cells, derive from Lgr5$^+$ crypt base columnar cells. Deletion of the adenomatous polyposis coli gene, a central regulator of Wnt signaling via its ability to target β-catenin for proteasomal degradation, in Lgr5$^+$ cells resulted in fast and progressive transformation of crypt-based cells (Barker et al. 2009). In vitro, Lgr5$^+$ stem cells form crypt-like structures independently of mesenchymal stromal cells, and Lgr5$^+$ cells remain at the apex of the stem cell hierarchy in these newly generated crypts (Sato et al. 2009). Hence, Lgr5 marks tissue stem cells of the intestine, which are, unlike other stem cells, rapidly cycling cells (approximately one division in 24 h) and hence potential targets for transformation in vivo.

Functionally, Lgr5 has long been termed an orphan receptor owing to the lack of knowledge of its cognate ligand. In 2011, it was revealed that Lgr5 is a receptor for the R-spondin family of stem cell factors (Carmon et al. 2012). The R-spondin family is composed of four members, which act synergistically with canonical Wnt ligands to strengthen downstream signals. Although involvement of Lgr5-coupled G proteins would have been expected, none of the classic processes such as calcium mobilization and cyclic AMP production were affected. Presently, the consensus is a model in which R-spondins/Lgr receptors enhance Wnt/β-catenin signaling via the formation of a so-called supercomplex with the plasma-membrane-associated co-receptor Lrp6 of the Wnt pathway (Carmon et al. 2012; Glinka et al. 2011). How this interaction eventually increases Wnt signaling remains unknown.

Thus, once more the Wnt signaling is affected as a central event in CSC regulation, and Lgr5 can be seen as a modulator of this pathway. On the basis of current knowledge, Lgr5 needs to be categorized as an indirect primary trait regulator because of its effects on Wnt signaling (Fig. 20.1).

The relevance to CSCs as a primary/secondary trait regulator is as follows:

- Communication with microenvironment
- Pluripotency and differentiation capacity (plasticity)
- Self-renewal

20.9 CD166

CD166 is a member of the immunoglobulin superfamily, whose synonyms are activated leukocyte cell adhesion molecule (ALCAM), KG-CAM, BEN/DM-GRASP, hematopoietic cell antigen (HCA), and neurolin (Ohneda et al. 2001). Expression of CD166 was described on normal hematopoietic cells, mesenchymal stem cells, neuronal cells, and stromal cells (Swart 2002). CD166 undergoes homotypic interactions and forms heterocomplexes with CD6. CD166 is

overexpressed in head and neck tumors, invasive melanomas, pancreatic carcinomas, and prostate carcinomas, and is an independent prognostic marker for some entities (Weidle et al. 2010). CD166 has, to the best of our knowledge, only been used for the enrichment of CSC from colon cancers and in glioblastomas (Kijima et al. 2012; Dalerba et al. 2007). Most of the information available on the function of CD166 in tumor cells relates to adhesive properties based on homotypic interactions. In vitro and in vivo homotypic interaction is counterregulated on shedding of the extracellular domain by the membrane-associated protease ADAM17/TACE (Rosso et al. 2007). Blocking of adhesion on treatment of cells with an antagonizing antibody resulted in enhanced migration of ovarian carcinoma cells, whereas the inhibition of TACE caused the opposite effect along with reduced wound-healing capacities in vitro. It is tempting to speculate that CSCs first express CD166 in order to allow the adherence of small numbers of CSCs at a first site in vivo to generate an initial tumor "seed" and avoid dispersion of cells before they have acquired migratory and invasive potential, and/or a critical minimal tumor size. When tumor cells acquire traits of invasiveness or proliferation, adhesive functions of CD166 can be obviated on shedding of the ectodomain (as exemplified in Fig. 20.2), which can be monitored in vivo as the presence of soluble CD166 in sera and ascites of tumor patients to a higher degree than in normal donors (Rosso et al. 2007). Similarly to CD44 and EpCAM, it is conceivable that shedding of the extracellular domain of CD166 is a prerequisite for regulated intramembrane cleavage of the intracellular domain via γ-secretase complexes. RIP would generate an intracellular domain of at least 34 amino acids, which is in size range comparable to that for CD44 and EpCAM. Additionally, CD166 displayed protective effects in breast carcinoma cells in vitro, correlated with the expression of the antiapoptotic molecule Bcl-2, and counteracted apoptosis in these cells (Jezierska et al. 2006). Whether this phenotype relies on adhesive or potential signaling capacities of CD166 remains entirely unknown. The most recent data point toward a function in the regulation of invasion and cancer progression in glioblastoma cells (Kijima et al. 2012). Here, suppression of CD166 expression promoted invasion without impacting on proliferation. The soluble form of CD166, on the other hand, fostered invasion and tumor progression.

The relevance to CSCs as a secondary trait regulator is as follows:

- Autonomy from tissue integrity
- Low apoptosis and high chemoresistance
- Migratory capacity and engraftment (metastasizing CSCs)

20.10 Conclusions and Outlook

The notion of heterogeneity among cancer cells in terms of their potential to induce tumors is an old one and actually dates back to the works of Cohnheim (1867, 1875) in the nineteenth century. From the middle of the twentieth century,

scientists followed up on the questions of whether tumor cells all have equal tumorigenic capacity and whether stem cells might be the origin of cancer. In 1960, Southam and Brunschwig (1960) performed autotransplantation experiments and revealed that large numbers of cells are necessary, and that engraftment of tumor cells is inefficient The idea of tissue stem cells being the "bad seed" was postulated in the following year (Till and Mc 1961). The first phenotypic, marker-based description of CSCs was reported by Lapidot et al. (1994) in work on AML that revealed $CD34^+/CD38^-$ AML cells as the CSCs in this entity using limiting dilution experiments in immunocompromised mice (Lapidot et al. 1994). Seminal work on the description of CSCs in solid tumors by Al-Hajj et al. (2003) showed that $CD44^+/CD24^-$ breast cancer cells have tumorigenic potential. Now, ten years later, the numbers of publications on CSCs have increased tremendously, with reports of CSCs in most if not all major cancer entities. However, the numbers of markers used for the enrichment of these tumorigenic cells remains surprisingly steady (Visvader and Lindeman 2008). In other words, the very same markers are in use in totally different tumor entities, with CD133 being a CSC marker in glioblastomas (Singh et al. 2004) and colon carcinomas (O'Brien et al. 2007; Ricci-Vitiani et al. 2007). In contrast, the same entity seems to include CSCs, which are characterized by different markers, as is the case for CD133 (O'Brien et al. 2007; Ricci-Vitiani et al. 2007) and CD44 (Dalerba et al. 2007) in colon cancers.

What have we learned from these findings?

Two assumptions must be made:

1. Tumor-initiating cells from different entities will acquire similar traits.
2. Plasticity is required for tumor-initiating cells to adapt to the differing conditions they encounter.

On the basis of these assumptions, the expression of common CSC markers might reflect the acquisition of such traits. If we assume that a subset of cells in a tumor have strongly enhanced tumor seeding potential and further assume t that genetic and epigenetic modifications are the basis for these intercellular differences, a more general concept can be postulated. According to this concept, CSCs require a minimal subset of functions, which can be provided to them by various combinations of reported CSC markers (exemplified in Fig. 20.1) and yet unknown proteins. The recurrence of CSC markers such as CD44, CD133, EpCAM, and ALDH, whose functions cover the basic requirements for self-renewal and differentiation capacity, supports this notion and speaks in favor of a general concept of CSC formation based on primary traits (Fig. 20.1, Table 20.1). A substantial number of these markers are regulated via Wnt signaling at the transcriptional level and on RIP at the functional level. Hence, after activation of the expression of CSC markers on deregulation of key signaling pathways, different phenotypes of CSCs can be modulated and smooth transitions thereof generated depending on the availability and activation status of essential components of RIP (Fig. 20.2). With knowledge of essential transcription factors required for reprogramming of induced pluripotent stem cells, the common overexpression of CSC markers such as EpCAM, which have the capacity to induce a subset of these

Table 20.1 Regulators of primary and secondary traits of cancer stem cells

Primary traits	
Self-renewal	EpCAM (Gonzalez et al. 2009; Ng et al. 2010); CD133 (Bauer et al. 2008); Lgr5 (Barker et al. 2009)
Pluripotency	CD44 (Bourguignon et al. 2009a, b, 2010); EpCAM (Gonzalez et al. 2009; Ng et al. 2010; Lu et al. 2010; Chen et al. 2011a); CD133 (Freund et al. 2006; Bauer et al. 2008; Kemper et al. 2010); CD24 (Morel et al. 2008); Lgr5 (Barker et al. 2009; Haegebarth and Clevers 2009; Sato et al. 2009)
Switch between quiescence and proliferation	CD44 (Pelletier et al. 2006)[a]; EpCAM (Maetzel et al. 2009; Munz et al. 2004; Maaser and Borlak 2008)[a]; CD47 (Sick et al. 2011); ALDH (Gudas and Wagner 2011)
Secondary traits	
Low apoptosis	CD44 (Zeilstra et al. 2008), CD47 (Sick et al. 2011); ALDH (Ma and Allan 2010)
Chemoresistance	CD44 (Zeilstra et al. 2008; Bourguignon et al. 2009b); ALDH (Ma and Allan 2010)
Communication with microenvironment	CD44 (Zoller 2011); CD133 (Freund et al. 2006); EpCAM (Litvinov et al. 1997); CD166 (Rosso et al. 2007)[a]; CD47 (Lindberg et al. 1993; Gao and Frazier 1994)
Autonomy from tissue integrity	CD44 (Bourguignon et al. 2010); EpCAM (Munz et al. 2004), Lgr5 (Sato et al. 2009)
Migration and engraftment	CD44 (Nagano and Saya 2004)[a] (Gunthert et al. 1991; Jin et al. 2006; Bourguignon et al. 2009b); EpCAM (Osta et al. 2004); CD133 (Hermann et al. 2007); CD24 (Baumann et al. 2005); CD166 (Rosso et al. 2007)[a]

ALDH aldehyde dehydrogenase, *EpCAM* epithelial cell adhesion molecule, *Lgr5* leucine-rich-repeat-containing G-protein coupled Receptor 5

[a]These functions have been demonstrated to depend on regulated intramembrane proteolysis.

central molecular switches, to promote proliferation, and to sustain pluripotency, appears consequential. Modulation of this expression will certainly impact on the capacities of CSCs, for example, resulting in the reported switch from an epithelial, proliferative status of CSCs to a more mesenchymal, migratory phenotype on downregulation of EpCAM.

In summary, a substantial number of markers for CSCs and normal stem cells share common features:

- They are Wnt signaling target genes (CD44, Lgr5, EpCAM).
- They display dual functions as cell adhesion molecules and signaling receptors (CD44, EpCAM, CD47, and possibly CD166).
- They are modulated in their function on ectodomain shedding and RIP (i.e., CD44, CD166, EpCAM).

Hence, co-regulation of expression and function of CSC markers by Wnt signaling and RIP, respectively, emerges as a potential common theme (Figs. 20.1, 20.2). Wnt signaling might therefore represent one of the key initial prerequisites for the priming of premalignant cells to become CSCs, which endows cells with a

panoply of adhesive and signaling properties, which may result in either communicative but rather quiescent cells ("adhesive phenotype") or dividing cells ("proliferative phenotype") (Fig. 20.2).

It is attractive to think of a specific inhibition of the expression/function of CSC markers, which are "drivers" in tumorigenesis, metastatic spread, and recurrence. Combinations of inhibitors of Wnt signaling and potent inhibitors of ADAM proteases and γ-secretase emerge as treatment options along with therapeutic antibodies. Additionally, interruption of central signaling cascades of CSC markers such as CD44, CD133, and EpCAM is a highly promising future approach.

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Chapter 21
Cancer Cell Dormancy: Potential Therapeutic Targets To Eradicate Cancer Cells Within the Niche

Jessian L. Munoz, Jacqueline M. Park, Sarah A. Bliss and Pranela Rameshwar

Abstract Breast cancer is one of the leading causes of cancer-related death in the USA. Metastasis and maintenance of breast cancer in the adult bone marrow could result in quiescence with resistance to chemotherapy. These changes are partly due to the interactions between cancer cells and the resident bone marrow cells, especially the cells residing close to the endosteum. Although the literature on cancer stem cells has exploded over the past few years, there is no clear indication that dormancy is exclusive to the stem cell subset of cancers. We discuss a role for the tissue microenvironment in cancer dormancy and also expand on the role of other stem cells. The information on cancer dormancy now has another significant role in medicine—specifically, in the field of stem cell therapy, in which cancer dormancy could be a potential confounder for safe treatment. This chapter discusses the interaction between cancer cells and mesenchymal stem cells. This area of discussion is particularly important considering the ongoing clinical trials with mesenchymal stem cells in which the treatments might be administered to individuals with undiagnosed cancers.

Keywords Breast cancer • Metastasis • Bone marrow cells • Mesenchymal stem cells • Cancer cells • Dormancy

21.1 Introduction

A major obstacle to effectively treat breast cancer involves the cancer cells' ability to acquire cycling quiescence for dormancy, thus evading antineoplastic treatment modalities. This is particularly important in bone marrow, which is an organ that seems to facilitate the quiescence of breast, and most likely, other cancer cells

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(reviewed in Rameshwar 2010). Despite aggressive treatment, and compliance with routine mammograms, breast cancer remains a clinical dilemma (Jemal et al. 2010). Breast cancer can resurge from the bone marrow after more than 10 years of remission, despite early detection (Green and Taplin 2003; Habeck 2000; Mansi et al. 1989; Naume et al. 2007; Riethdorf and Wikman 2008). Even in undiagnosed cancer, occult cancer cells can exist for more than a decade before the cancer is clinically detected (Talmadge 2007). In several cases of resurgence, the bone marrow has been attributed as the source of tertiary metastasis (Pantel and Alix-PanabiFres 2010). This chapter will focus on bone marrow; however, the information can be extrapolated to any organ where breast or other cancer cells lie as dormant cells.

Dormant breast cancer cells are expected to be at the phase of cycling checkpoint. This quiescent phase will provide cancer cells with a survival advantage because noncycling cells are generally insensitive to common anticancer agents, whereas proliferating cells are efficiently targeted by anticancer agents (Corcoran et al. 2008; Moharita et al. 2006; Rao et al. 2004). This chapter will discuss the mechanisms by which breast cancer cells attain dormancy since this could explain cancer resurgence. The discussion will also include the possibility of treatment resistance since this will also explain dormancy in tissues.

21.2 Breast Cancer Dormancy: More Questions Than Answers

It is unclear if cancer cells attain dormancy through mechanisms by external factors from a microenvironment or if dormancy is caused by an intrinsic property of the cancer cells. In the case of intrinsic properties of dormancy, the cancer cells will resist treatment regardless of the organ or tissue. The dormant cells might be the long-existing cells in an organ such as the bone marrow niche. If breast cancer dormancy is caused by intrinsic properties, then one can assume that these cells are similar to other dormant breast cancer cells that might be generated through changes caused by factors and/or cells in the bone marrow microenvironment. Regardless of the mechanism, the dormant subsets in bone marrow, and perhaps other organs, are likely to be the sources of tertiary metastasis. Eradication of these dormant cells will depend on understanding their differences and/or similarities, as well as their overlap with endogenous stem cells.

There are several questions regarding breast cancer dormancy in the bone marrow microenvironment. First, is there a specific location for dormant cancer cells in the bone marrow? Second, does dormancy occur in a special subset of cancer cells? Third, what could explain the failure of the bone marrow, an immune organ, to clear residual cancer cells? Fourth, what are the mechanisms by which cancer cells take advantage of the bone marrow microenvironment to evade treatment and detection? Last, and perhaps most important, what are efficient methods to target cancer cells while they reside in the bone marrow microenvironment?

An understanding of the mechanism by which breast cancer cells acquire dormancy in bone marrow first requires the identification of the subset(s) of these cells. Although there are widespread discussions on cancer stem cells with reports on their phenotype, the literature remains open for further investigations. If there is heterogeneity of breast cancer cells in a patient, the cancer stem cells in lung or brain might be different from those in bone marrow. If this is the case, does this indicate that there are different cancer stem cells in the bone marrow, or do the variations transition to a common type when the cancer cells contact the bone marrow microenvironment? These arguments/questions underscore the challenges that researchers must face in future treatment of dormant breast cancer cells in the bone marrow microenvironment.

21.3 Bone Marrow Microenvironment in Cancer Dormancy

The literature provides experimental evidence that underlies the assumptions regarding the facilitating roles of distinct regions of the bone marrow for survival of dormant cancer cells (Habeck 2000; Mansi et al. 1989; Naume et al. 2007; Riethdorf et al. 2008). It is likely that dormant cancer cells will be found close to the endosteal region, where they form gap-junctional intercellular communications (GJICs) with hematopoietic supporting cells, stroma, and perhaps osteoblasts (Calvi et al. 2003; Chow et al. 2001a, b; Dorshkind 1990; Jang and Sharkis 2007; Kiel and Morrison 2008; Muller-Sieburg and Deryugina 1995; Taichman and Emerson 1994; Taichman and Emerson 1998). Consistent with this assumption is a report revealing noncycling breast cancer cells close to the endosteum of a xenogeneic mouse model, whereas proliferating cancer cells are observed within the cellular compartment of the bone marrow (Rao et al. 2004). The microenvironment of the bone marrow makes this organ "inviting" for breast cancer cells, where they take advantage of the microenvironment for their survival. On crossing the blood vessels from the periphery to bone marrow, the breast cancer cells encounter mesenchymal stem cells (MSCs) on the abluminal side of blood vessels, where they interact via CXCL12–CXCR4 (Corcoran et al. 2008). This initial interaction with MSCs is interesting in that these stem cells are immune suppressors and are capable of conferring immune protection on cancer cells (Potian et al. 2003). Investigation of the role of MSCs in breast and other cancers is a rapidly growing field of study, and understanding their role will potentially lead to advancements in treatments. At this time, however, it is difficult to predict the direction in which MSCs will lead to new treatments. This is mainly due to the ubiquitous presence of these stem cells. More importantly, it is believed that pericytes, which surround blood vessels, are MSCs, or at least share functions and phenotypic markers.

Dormancy of breast cancer cells during remission or before detection is not necessarily accompanied by bone marrow failure, suggesting that hematopoiesis remains unaffected by the same mechanism responsible for cancer cell dormancy. In fact, experimental studies show GJIC between stroma and cancer cells

without affecting hematopoietic activity (Moharita et al. 2006). It is paramount for research to continue in this area to address the mechanisms by which the microenvironment of bone marrow can protect cancer cells without affecting hematopoietic activity. Information on this topic could lead to targeting of cancer cells without disruption of the hematopoietic system.

The integration of breast cancer cells among bone marrow cells is expected to involve cytokines, which are major growth factors required for hematopoietic support. However, microRNAs (miRNAs) and RNA-binding proteins are evolving as significant players in the already complex networks among cancer cells and endogenous bone marrow cells. It will be difficult to use these networks to develop targets because there are overlaps with the hematopoietic system. A major challenge in the past that continues to hinder progress lies in targeting dormant cancer cells while protecting cells within the hematopoietic system.

The formation of functional GJIC between breast cancer cells and bone marrow stroma suggests that small molecules could be exchanged through the GJIC. Studies in this area are required as this could be paramount to the quiescent phase of the cancer cells. Although stromal cells have been studied, osteoblasts could also be relevant for the generation of GJIC since the growing literature suggests that osteoblasts are significant for hematopoiesis. Furthermore, if miRNAs are relevant to breast cancer cell dormancy, their roles will be complex and not limited to cell cycle checkpoints, but will perhaps involve mechanisms of protection during chemotherapy and against other toxic agents.

MicroRNAs (miRNAs) can pass through gap junctions from stroma to cancer cells, and this facilitates cell cycle quiescence. Among the miRNAs transferred are those specific for decreasing the levels of stromal-cell-derived factor 1 (CXCL12) (Lim et al. 2011). Further studies are required to understand how the levels of CXCL12 are linked to its receptor, CXCR4, and gap-junctional protein expression. The current focus is on connexin 43 because highly metastatic breast cancer cells have been reported to lack this connexin although they express other members of the family (Cher et al. 2006). One of the most studied triple-negative breast cancer cells, MDA-MB-231, has been reported to be CXCL12-null (Kang et al. 2005). The evolving information on breast cancer subsets should revisit information such as the production of CXCL12 as this might provide insights into cancer dormancy.

The location of dormant breast cancer cells close to the endosteal region adds to the complexity in developing strategies to treat cancer cells in regions close to the bone. This area is also the “home” of hematopoietic stem cells, which are sensitive to the toxic effects of drugs, cancer drugs included. The dose of a drug for cancer treatment is limited to the concentration that shows no toxic effect on hematopoietic stem cells. Thus, in the process of protecting hematopoietic stem cells, the treatment could be, unknowingly, protecting the regional breast cancer cells that are likely dormant.

The impact of studies to understand how breast cancer cells adapt in the bone marrow microenvironment can be appreciated if one revisits the failed autologous transplantations of the hematopoietic system for breast cancer patients. If the quiescent breast cancer cells are located within the endosteal region, forming GJIC

with stroma, then high-dose chemotherapy will be inadequate to eliminate the cancer cells in this region. In the past, autologous transplantation with hematopoietic stem cells was performed in a setting in which dormant cancer cells existed.

Failed clinical trials can also provide insights into the targeted disease. Autologous transplantation with hematopoietic stem cells for treatment of breast cancer revealed substantial information on the biology of patients' bone marrow. The cohort of patients with inflammatory breast cancer responded positively to autologous hematopoietic stem cell transplantation. This raises the question of differences in the cancer cells, based on their functions and hormone expressions. The questions posed in this chapter, if answered, could lead to the identification of new targets to translate the science to patients, ultimately to apply innovative methods to target dormant breast cancer cells without causing harm to the endogenous hematopoietic stem cells in the same region. On the other hand, if bone-marrow-derived MSCs are critical to the dormancy, an understanding of the mechanism by which these stem cells facilitate dormancy could be relevant to future treatments, either as absolute treatments or for prevention. The discussion is relevant to all phases of breast cancer since dormant cancer cells could be present during periods of overt metastasis, during remission, and also before detection since the entry of cancer cells into bone marrow might occur long before detection.

21.4 Cancer and Other Stem Cells

Any discussion on cancer biology, directly or indirectly, includes stem cells such as cancer stem cells or the related tumor-initiating cells and MSCs. Also of importance are tissue-specific stem cells since they could be the source of the original tumor. The existence of cancer stem cells is mostly accepted by the scientific community (Coombe 1996; Korkaya and Wicha 2010). The future of this field, however, could face problems depending on how academia, biotechnology companies, and pharmaceutical companies use the information for cancer eradication. Scientists in academia are focused on basic science to identify the hierarchy of cancer cell subsets; biotechnology companies are also involved in basic science, but these companies are mindful of commercial benefits. Pharmaceutical companies, on the other hand, are interested in targeting cancer cells to achieve cell death. Although on the surface this appears to be ideal, it could also be a dilemma for targeting cancer stem cells.

When one discusses cell death, it is always unclear what particular cancer cell subsets are the targets. If highly replicating cells are the intended targets, this could lead to an ethical problem since this will add a new drug to achieve an outcome similar to that of past therapies. The ideal scientific outcome is to eradicate cancer at the level of stem cells. The answer to this could be simple since many genes linked to self-renewal and pluripotency have been identified. However, these same genes are also expressed in all stem cells. Perhaps scientists might consider a balance of benefits with regard to eliminating cancer cells with reduced toxicity. However, this balance might be more difficult to achieve since the literature,

for the most part, indicates a low frequency of cancer stem cells. This implies that the requirement to eliminate cancer stem cells might also result in elimination of healthy stem cells, which are also present with low frequency, resulting in overt toxicity. To achieve curative treatment requires some convergence and divergence of the goals of academia, biotechnology companies, and pharmaceutical companies to reduce the number of confounders facing the ultimate beneficiaries, the patients.

21.4.1 Mesenchymal Stem Cells

MSCs are linked to the function of cancers. This section briefly discusses the biology of these stem cells. There are ongoing experimental studies and clinical trials using stem cells, including MSCs, to deliver drugs to tumors (Ghaedi et al. 2011; Meyerrose et al. 2010; Porada and Almeida-Porada 2010; Saito et al. 2011; Song et al. 2010; Zhao et al. 2008, 2012). There are several advantages of using MSCs, but the main advantage is their ability to be delivered across an allogeneic barrier (English and Mahon 2011). MSCs are available as an off-the-shelf source for immediate delivery, and this is primarily due to the cells' immune suppressor functions. However, a subset of MSCs express the major histocompatibility antigen, which can mediate the cells' ability to function as antigen-presenting cells, and also to cross-present antigens (Chan et al. 2006; Francois et al. 2009; Romieu-Mourez et al. 2007). These immune functions of MSCs could also compromise their safety. However, this chapter is focused on the potential confounders of cancer stem cells and will therefore not discuss the possible confounder of MSCs switching to immune-enhancer roles. Similarly, although the literature indicates that MSCs, unlike embryonic stem cells, are less likely to form tumors, this topic will not be discussed here.

To reiterate, dormancy of breast cancer remains a crucial unresolved problem. It is necessary to identify and to eradicate the dormant cancer cell population and/or to intervene and to change the microenvironment to prevent dormancy. This will require advanced studies on the hierarchy of cancer cells and determination of how these cells are regulated by the microenvironment. Thus, it is expected that the hierarchy is limited not only to phenotype, but also to function. Studies should be done to determine if the reported phenotypes for cancer stem cells are indeed robust, such as $CD44^{+}/CD24^{-}$ for breast cancer stem cells (Al-Hajj et al. 2003).

If dormancy begins at the primary site in the mammary gland, the challenge could be limited to the intrinsic property of the cancer cells. On the other hand, if dormancy is dictated by the microenvironment, the dormant cancer cells will differ, depending on the location of the cells. An important area of ongoing research is the role of MSCs, which can produce growth factors and also protect from immune responses.

The data pertaining to dormancy by GJIC with stroma needs to be examined for drug resistance. Studies are also required to determine if the cells that failed to form GJICs can behave differently in response to chemotherapy. If the

stem cells are mostly involved in GJIC and in dormancy, differentiation could be an efficient method to reverse dormancy. This will result in drugs that induce the differentiation of the stem cells to make them chemosensitive. Recent literature has reported on "dedifferentiation" of noncancer stem cells to cancer stem cells, further supporting intervention with drugs to induce differentiation (Chaffer et al. 2011). Last, an important point is the ongoing questions on the usefulness of removing lymph nodes (Giuliano et al. 2011; Kawada and Taketo 2011; Riethdorf et al. 2008). The question then is whether the dormant cancer cells can bypass lymph nodes, and if they can, whether negative lymph nodes are indicative of non-metastatic cancer cells. These are important questions for future studies since the answers will result in new targets to reverse dormancy for cancer eradication.

Scientists in academia rely on government agencies or nonprofit foundations to fund their research. In most cases, academicians, without a business background, do not appreciate the lack of resources in comparison with pharmaceutical companies, which possess an impressive infrastructure and expertise in drug development. In these scenarios, good ideas could be curtailed owing to lack of resources. The scientific dilemma, as already mentioned, is for pharmaceutical companies to accept the challenge of developing a drug that acts against cancer stem cells, despite the obvious toxicity. The genes that maintain pluripotency in cancer stem cells are also expressed in stem cells, which are ubiquitous. Other targets that seem almost ideal, on the basis of the experimental outcomes, have also been identified. Despite this, the prediction of toxicity seems obvious. Recently, miR-34a was identified as a potential target to eliminate prostate cancer stem cells (Liu et al. 2011). The premise is that miR-34a will suppress the expression of CD44 on the cancer stem cells to prevent metastasis. The advantage is the development of novel RNA therapy. The disadvantage is potential overt toxicity since the ligand of CD44, hyaluronic acid, is a constituent of the extracellular matrix of the bone marrow microenvironment that supports hematopoiesis (Coombe, Coombe 1996).

Research studies have identified cytokines as potential targets to eliminate cancer stem cells (Ginestier et al. 2010). In other investigations, cytokines were implicated as regulators of other genes to establish quiescence of cancer cells in bone marrow, where it is difficult to target cancer cells (Moharita et al. 2006; Oh et al. 2004). Cytokines might be attractive targets owing to the presence of redundant functions. Specifically, blocking a particular cytokine is unlikely to cause deleterious effects on healthy cells, since there are other cytokines with similar effects. The same argument regarding functional redundancy of cytokines can be extrapolated to cancer stem cells, but the effects could be nullified by other cytokines. Specifically, although a particular cytokine might appear to be an attractive target with regard to cancer stem cells, the stem cells might produce other cytokines to counteract the targeted molecule. Therefore, this subject requires further investigation to determine the effectiveness of targeting cytokines. In addition to functional redundancy, cytokines exhibit pleotropic effects, in which one cytokine can have effects on multiple downstream targets. Thus, it may be difficult to achieve therapeutic specificity when targeting one cytokine in an attempt to eliminate cancer stem cells.

Contributing to the complex issues of cancer stem cells is the role of MSCs in cancer biology. MSCs can protect against as well as support cancer growth (Corcoran et al. 2008; De et al. 2010; Feng and Chen 2009; Greco and Rameshwar 2008; Mishra et al. 2008; Momin et al. 2010; Patel et al. 2010; Rameshwar 2010; Riggi et al. 2010). The most obvious target of cancer support by MSCs is a direct target. However, targeting of MSCs could be the most toxic method, partly because of these stem cells, also referred as pericytes, which surround blood vessels (Feng et al. 2010). The supporting role of pericytes with regard to blood vessels appears to be an attractive target to eliminate angiogenesis. However, specificity with regard to target tumor-associated blood vessels will be difficult to achieve and this method is likely to result in overt collapse of most, if not all, blood vessels.

The ubiquitous presence of MSCs makes them relevant to tumor biology. It is ironic that the information regarding the role of MSCs is overwhelming, perhaps more so than that in the field of cancer stem cells. Yet, targeting MSCs might be more of a challenge owing to the cells' essential endogenous functions. Among other functions, the immune-suppressive roles of MSCs are relevant. This property of MSCs might partly explain the reduced enthusiasm for immune therapy for tumors. The information on MSCs should allow scientists to reexamine the field of immune therapy for cancers and to examine methods that take advantage of the unique immune properties MSCs possess in order to improve cancer therapy.

Although in this chapter we briefly discuss the issue of cancer and its link to MSCs, it is clear that no single entity would be able to develop targets. To overcome scientific and ethical dilemmas associated with cancer and MSCs will require collaborations among academia, biotechnology companies, and pharmaceutical companies. Ethical problems concerning the targeting of stem cells could arise owing to the foreseeable toxicity with regard to endogenous stem cells, which in turn means that development of a drug will require a prolonged period of time. These ethical problems are also the scientific issues that need to be addressed in long-term robust research studies. In summary, the question of targeting cancer stem cells as well as the supporting MSCs in cancer cannot be achieved by one entity, and requires a partnership among academia, biotechnology companies, and pharmaceutical companies. This could indirectly allow government and private funds to be combined in the attempt to eradicate a disease among all humans, regardless of geographical location.

21.4.2 Cancer Stem Cells: Problems for Other Stem Cells

The definition of cancer stem cells is not different from that of any other stem cells. Thus, it is expected that a cancer stem cell will be present at a low frequency, self-renew, and initiate and support tumors (Clarke et al. 2006). Reports indicate that cancer stem cells may be radioresistant (Krause et al. 2011). Unlike the detailed hierarchy for hematopoietic stem cells, similar steps in lineage

commitment have not been elucidated for other stem cells, including cancer stem cells. Tumor-initiating cells have traditionally been referred to as dormant cancer cells, which are considered to be responsible for cancer resurgence (Clarke et al. 2006; Democheli et al. 1994; Nava 2010). As the field of cancer stem cells begins to develop into a hierarchy, the field of stem cell therapy will benefit because decisions can be made on which cancer cells will be affected by other stem cells.

The major problem that is envisioned for stem cell therapy is linked to undiagnosed cancers. It is thought that approximately 30 % of middle-aged adults have an undiagnosed cancer (Folkman and Kalluri 2004). As would be expected, stem cell treatment would be likely indicated for a similar cohort of individuals. Thus, it will be paramount to understand how any implanted stem cell can affect undiagnosed cancers, and this requires in-depth experimental analyses.

A difficult question to answer is whether stem cell therapy should be designed with strategies to retain the dormant cancer cells in their quiescent phase. Answers to this question are not simple because MSCs have varied effects on tumors. MSCs are currently in clinical trials to directly deliver drugs to target tumors. The main disadvantage of using MSCs for this purpose is their ability to support cancer growth and progression (Greco et al. 2011; Grisendi et al. 2011). Although the use of MSCs for targeted therapy is an excellent method, the approach could subsequently protect cancer stem cells. It is expected that the drugs will target the rapidly dividing cancer cells. However, if the drugs cannot target the stem cell subset, MSCs could protect these cells to encourage dormancy. Perhaps, MSCs could be protective by inducing and expanding immune-suppressor regulatory T cells and/or by delivering small RNA either through the formation of gap junctions or through exosomes (Gregory et al. 2011; Lim et al. 2011). There are reports supporting the presence of small RNA within exosomes released from tumor cells. In general, stem cells are not metabolically active cells and are therefore expected to be “rich” sources of RNA that could be delivered to tumor cells. Cancer stem cells could benefit from the proximity of MSCs to establish GJIC, which can facilitate the passage of small miRNA that benefits a dormant phase of the cancer cell (Lim et al. 2011; Matuskova et al. 2010).

21.5 Conclusion

We have discussed cancer dormancy and highlighted the possibility that cancer stem cells could be candidates as dormant cells. We have also discussed that the field of dormancy has implications for future treatment with other stem cells, in particular MSCs and other similar cells. Figure 21.1 delineates the metastasis of cancer cells to different organs and demonstrates a role for MSCs in the biology of cancer cells. MSCs can come in contact with circulating cancer stem cells or migrate to the site of dormancy. Future studies will determine how stem cell therapy could be given without resurgence of existing cancer. Ideally, it will be better to develop targets to eradicate the existence of dormant cancer cells prior to

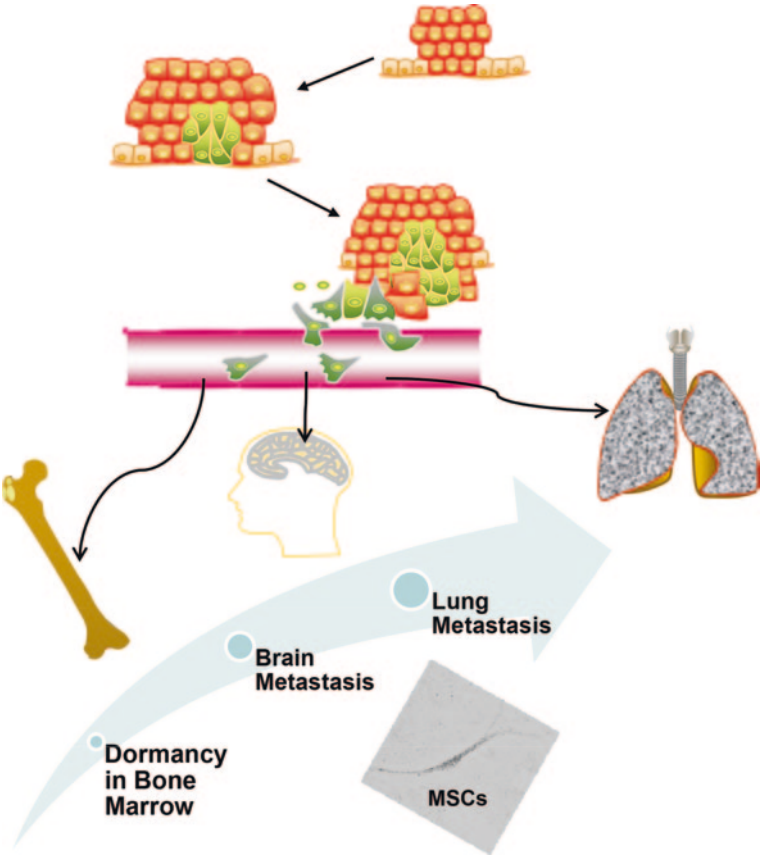

Fig. 21.1 The development of transformation at a primary site (*top right*). The cells acquire additional mutations and begin to replicate (*top left*). As the transformed cells proliferate, they become invasive and metastasize to different organs such as bone marrow, brain, and lungs. Mesenchymal stem cells (*MSCs*) facilitate the metastasis, although the mechanism of this process continues to be a subject of investigation

stem cell therapy. In this regard, both fields overlap. Dormancy of cancer has been described, although it is yet to be determined if the cancer stem cells are responsible for a dormant phase. Regardless, robust research is required to understand how stem cells such as MSCs affect preexisting cancer stem cells and their progenies. There is intensive research to develop methods to prevent the transition of dormant cancer cells into rapidly growing cells. However, these strategies propose retaining the dormant cells in the individuals. Perhaps this is a good strategy since 30 % of individuals have undiagnosed occult cancer. On the other hand, this could be a problem if transplanted MSCs can support the transition of dormant cancer cells into aggressively dividing cells. It is, therefore, crucial to carefully review the issues regarding cancer stem cells and MSCs for safe treatment.

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Chapter 22
Modulating Apoptosis in Cancer Therapy with Ultrasound and High-Intensity Nanosecond Electric Pulses

Tinghe Yu, Minghe Wu, Ping Huang and Lina Hu

Abstract Ultrasound can induce apoptosis and enhance apoptosis attributable to anticancer drugs, in which cavitation plays the leading role. Cavitation leads to the production of reactive radicals and shear forces, which insult DNA and mitochondria, initiating apoptosis via the cytochrome *c*–caspase 3 pathway. Cavitation permeabilizes the cell membrane, thereby increasing the intracellular drug level and enhancing the efficacy of the cytotoxic drug. Ultrasonically chemotherapeutic sensitization is effective in both chemosensitive and chemoresistant cancer cells. The biological responses to high-intensity electric pulses are dependent on the voltage applied and the pulse length. Nanosecond electric pulses can pass through plasma and nuclear membranes to create sufficiently high voltages in the cytoplasm and in the nucleus with intact plasma and nuclear membranes, thereby impacting mitochondria or DNA, resulting in apoptosis. Nanosecond electric pulses may induce apoptosis via the intrinsic or the extrinsic pathway, and electrical and mechanical mechanisms may be involved. Both ultrasound and electric pulses can be delivered precisely into preselected tissues, so these two techniques can be developed for targeted cancer therapy.

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22.1 Introduction

Cancer therapies such as cytotoxic agents and radiation usually deactivate malignant cells by inducing apoptosis, and the malfunction/insufficiency of apoptosis plays an important role in therapeutic resistance (Girdhani et al. 2005; Meiler and Schuler 2006; Limtrakul 2007). Sensitizing apoptosis (i.e., modulating the threshold for apoptosis) can improve the therapeutic efficacy.

Strategies have been developed to modulate apoptosis, thereby enhancing therapy or overcoming therapeutic resistance. As nondrug techniques, use of ultrasound and use of high-intensity electric pulses have recently been introduced to modulate apoptosis in cancer treatment. Insonation can induce apoptosis, and enhance apoptosis attributable to anticancer drugs (i.e., ultrasonic chemosensitization). The modality is effective in both chemosensitive and chemoresistant cells. The biological effects of high-intensity electric pulses are dependent on the voltage applied and the pulse length. Electric pulses with a duration of nanoseconds can pass through cellular and nuclear membranes, thus producing sufficiently high voltages in the cytoplasm and nucleoplasm, which induces apoptosis. These two techniques are briefly discussed in this chapter.

22.2 Ultrasound

22.2.1 Biological Effects of Ultrasound

Ultrasound induces thermal and nonthermal (mechanical effect and cavitation) effects in an insonated medium. Ultrasound heats tissues, leading to a temperature rise (ter Haar 2007; Yu and Huang 2011). Cavitation results in localized increases in temperature (10^4–10^6 K) and pressure (10^4 atm), thereby generating microjetting, microstreaming, and free radicals (Paliwal and Mitragotri 2006; Stride and Coussios 2010). These effects result in structural and/or functional changes in tissues. A specific ultrasonic effect usually plays the leading role in a specific biological response.

The biological effects are dependent on the properties of the ultrasound (intensity, frequency, wave mode, and exposure duration) and the tissues. Usually, a lower frequency favors the occurrence of cavitation, and a higher frequency favors heat deposition. The tissue type is a determinant of the biological effects; thus, biological responses differ between tissue types. It is more difficult to induce cavitation in dense tissues than in loose tissues, which can be overcome by the introduction of sonosensitizers and microbubbles. They enhance cavitation by

decreasing the cavitation threshold (the critical intensity for inducing cavitation at a given frequency). Ultrasound intensity attenuates exponentially with increasing depth in tissues [$I_x = I_0\exp(-\mu x)$, where I_0 is the intensity emitted, I_x is the intensity in the area of interest, μ is the attenuation coefficient, and x is the distance from the ultrasound source to the target area], suggesting that it is theoretically reasonable to use the intensity in situ when exploring biological effects (ter Haar 2001). However, the texture of tissues is very heterogeneous and biological ultrasound operates within the range of nonlinear acoustics; thus, it is impossible to predict the behavior of ultrasound in tissues (particularly in vivo) (Fry 1993).

Ultrasonic therapy applies acoustic effects. A therapeutic modality mostly uses one type of ultrasonic effect. Ultrasonic hyperthermia (below 50 °C) and high-intensity focused ultrasound therapy (about 56 °C) use heat. Cavitation plays the leading role in sonochemotherapy, sonodynamic therapy, and gene delivery (Lou et al. 2011). These can be used to select the insonation parameter and to monitor treatment. Ultrasound therapy is a noninvasive procedure as it can be focused on the target volume within the body without harming overlying and surrounding tissues.

22.2.2 Inducing Apoptosis

Ultrasound-induced apoptosis in leukemia cells is manifested by morphological changes and DNA breakages. Apoptosis was p53-dependent in M1/2 cells and p53-independent in HL-60, K562, and U937 cells (Ashush et al. 2000). Cavitation damages the membrane, nucleus, and organelles, thereby inducing apoptosis. It was found that p53-positive cells were more sensitive than p53-negative cells to ultrasound-induced apoptosis (Abdollahi et al. 2004). The activation of caspase 3, collapse of the mitochondrial membrane potential, and a decrease of the ratio of Bcl-2 to Bax occurred in K562 cells when apoptosis was induced with ultrasound (Lagneaux et al. 2002). The data show that insonation triggers apoptosis via the mitochondrial pathway. The apoptosis rate was increased with the introduction of cavitation-enhancing microbubbles, and decreased by the free radical scavenger histidine or mannitol (Lagneaux et al. 2002; Feril et al. 2003). Free radicals resulting from cavitation therefore play important roles in apoptosis. Indeed, an increase of the level of intracellular reactive oxygen species was detected in insonation-induced apoptosis (Honda et al. 2004; Xiang et al. 2011). Cavitation decomposes water molecules, leading to the formation of hydroxyl radicals, which initiate subsequent oxidative and peroxidative reactions producing many kinds of reactive oxygen species (Kardos and Luche 2001). These reactive radicals impair the cellular nucleus and mitochondria, thereby triggering apoptosis.

Apoptosis can be realized by the cytochrome *c* dependent or cytochrome *c* independent pathway; in the former pathway, the release of cytochrome *c* from mitochondria triggers apoptosis, and caspase 9 plays a critical role; caspase 8 is involved in the latter pathway; caspase 3 is the common downstream executioner enzyme in both pathways (Jiang and Wang 2004; Plati et al. 2008). The release of

cytochrome *c* and activation of caspase 3 were confirmed in insonated Jurkat and Raji cells, indicating involvement of the cytochrome *c*–caspase 9–caspase 3 apoptosis pathway (Firestein et al. 2003). Apoptotic bodies were detected in chemoresistant COC1/DDP human ovarian cancer cells, but with a decrease of the level of cytochrome *c* in the cytosol (Yu et al. 2005a). Caspase 8 was activated in hematoporphyrin–sonodynamic therapy against S180 cells (Tang et al. 2008). Insonation, therefore, may induce apoptosis independently of mitochondria.

Apoptosis is a very complicated programmed procedure. Ultrasonic apoptosis was explored from the perspective of genes/proteins. Insonation upregulated the expression of p53, p21, HMOX1, JUN, heat shock protein, ferritin and Bid, and decreased the levels of Bcl-2 and superoxide dismutases (Table 22.1). The data suggest that ultrasound induces oxidative damage, triggering cell apoptosis. Reactive oxygen species attributable to intracellular cavitation insult cells (particularly mitochondria and DNA). Those active molecules can deactivate the scavengers (superoxide dismutase), thereby decreasing the cells' protective capacity and enhancing free-radical-induced cellular damage. Shear forces attributable to cavitation split DNA stands, and irreparable damage induces apoptosis (Furusawa et al. 2012). Cavitation, therefore, is the determinant for ultrasonic apoptosis (Fig. 22.1).

22.2.3 Sensitizing Apoptosis Attributable to Anticancer Drugs

Ultrasound (1.0 MHz, 100-Hz pulse repetition frequency with 10 % duty cycle) enhanced apoptosis attributable to doxorubicin in U937 lymphoma cells. Doxorubicin alone led to an apoptosis rate of 3.0 %; the rate was increased to 6.5 % and 16.2 % at 0.3 and 0.5 W/cm^2 insonation, respectively (Yoshida et al. 2008). A higher level of caspase 3 was detected in colon 26 cells when ultrasound (1 MHz, 1.0 W/cm^2) was applied in cisplatin treatment, demonstrating the sensitization of apoptosis (Watanabe et al. 2008). Ultrasound can enhance the cytotoxicity of mitomycin, paclitaxel, 5-fluorouracil, cytosine arabinoside, nitrogen mustard, diaziquone, bleomycin, cyclophosphamide, thiotepa, or bortezomib (Yu et al. 2006; Poff et al. 2008). Enhancement of apoptosis is involved in the mechanisms because (1) insolation alone produced no or very slight cytotoxicity in those trials and (2) an anticancer drug usually deactivates cells by inducing apoptosis.

Ultrasound treatment overcomes adriamycin or cisplatin resistance in cancer cells (ovary, liver, and colon) (Yu and Zhang 2010). The application of noncytotoxic insonation increased the apoptosis rates attributable to adriamycin in chemoresistant $SKOV_3$/ADR ovarian cancer cells (Yu et al. 2004). The findings indicate that enhancing apoptosis plays an important part in ultrasound-mediated chemosensitization.

The mechanisms of ultrasound-enhanced apoptosis have been explored. Cavitation damages the cell membrane, thereby improving the permeability, which favors the influx of cytotoxic drugs, resulting in a higher intracellular drug level (Yu and Zhang

Table 22.1 Change pattern of apoptosis-related molecules after insonation

Cell	Insonation	Upregulation	Downregulation	Reference
TK6 lymphoblast	0.68 MHz 1.5 MPa 1 Hz (pulse)	p53 p21/waf Thy-1 (CD90) H-ferritin	Bcl-2 Superoxide dismutases	Abdollahi et al. (2004)
U937 lymphoma	1.0 MHz 3.6 W/cm^2	FTH1 AUH JUN EST HMOX1	v-*myb* Cathepsin G	Tabuchi et al. (2002)
Molt-4 leukemia	1 MHz 100 Hz (pulse) 0.3 W/cm^2	HSPA1B BAG3 HSPA6 DNAJB1	IDI1 HMGCS1	Tabuchi et al. (2007)
U937 lymphoma	1 MHz 100 Hz (pulse) 0.3 W/cm^2	JUN FOS FOSB HMOX1 HSPs SERPINE1 RELB DUSP1 KLF4 KLF6		Furusawa et al. (2010)
SMMC-7721 hepatocarcinoma	1.2 MHz 3.0 W/cm^2	HSP70 Bip/GRP78 HSP40 p53 Bid OSR1 Heme oxygenase 1 NADPH-adrenodoxin oxidoreductase NADH-ubiquinone oxidoreductase	Bcl-2 Prohibitin Mitofilin VDAC1 Peroxiredoxin 4 Ubiquinol–cytochrome *c* reductase complex core protein 1	Feng et al. (2010)

FTH1 ferritin, heavy polypeptide 1, *AUH* AU RNA-binding protein/enoyl-coenzyme A hydratase, *v-myb* v-*myb* avian myeloblastosis viral oncogene homologue, *JUN* v-*jun* sarcoma virus 17 oncogene homologue, *BAG3* Bcl 2-associated athanogene 3, *DNAJB1* DnaJ (heat shock protein 40) homologue, subfamily B, member 1, *HSPA1B* heat shock 70 protein 1B, *HSPA6* heat shock 70 protein 6, *IDI1* isopentenyldiphosphate Δ-isomerase 1, *HMGCS1* 3-hydroxy-3-methylglutaryl-coenzyme A synthase 1, *FOS* v-*fos* FBJ murine osteosarcoma viral oncogene homologue, *FOSB* FBJ murine osteosarcoma viral oncogene homologue B, *HMOX1* heme oxygenase 1, *HSP* heat shock protein, *SERPINE1* serpin peptidase inhibitor, clade E, member 1, *RELB* v-*rel* reticuloendotheliosis viral oncogene homologue B, *DUSP1* dual-specificity phosphatase 1, *KLF4* Krüppel-like factor 4, *KLF6* Krüppel-like factor 6, *Bid* BH3-interacting domain death agonist, *OSR1* serine/threonine protein kinase oxidative-stress-related protein 1, *NADPH* reduced nicotinamide adenine dinucleotide phosphate, *NADH* reduced nicotinamide adenine dinucleotide, *VDAC1* voltage-dependent anion-selective channel protein 1

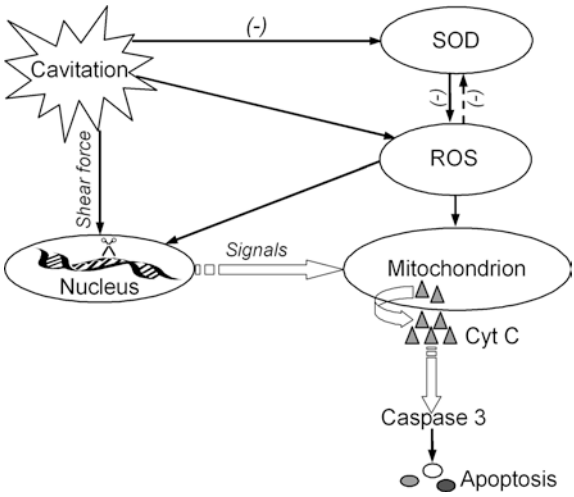

Fig. 22.1 Cavitation-induced apoptosis. Ultrasonic cavitation leads to the production of reactive oxygen species (*ROS*), which insult mitochondria, initiating apoptosis. Free radicals and shear force due to cavitation cleave DNA; irreparable damage triggers the signal pathway of apoptosis, which results in the release of cytochrome *c* (*cyt C*) from mitochondria, initiating cell death. Cavitation can deactivate the surrounding scavenger enzymes such as superoxide dismutase (*SOD*), thereby decreasing the cells' protective capacity

2010). The efficacy of a drug depends on the peak concentration and the area under the concentration–time curve. Ultrasound, therefore, impacts on the pharmacokinetics profile, enhancing the efficacy of an anticancer agent. Cancer cells were exposed to adriamycin followed by noncytotoxic insonation, and the survival curves were evaluated with the mathematic model $S = 1 - [1 - \exp(-D/D_0)^N]$, where S is the survival rate, D_0 is the dose of a drug required to give an S value of 0.37, and N is the number of intracellular sensitive sites. D_0 and N are cell-specific intrinsic parameters reflecting the cell's sensitivity to a cytotoxic stimulus. Both D_0 and N were altered by insonation, thereby upregulating the cells' sensitivity to a drug (i.e., ultrasonic chemosensitization) (Yu et al. 2005b). The increase of the apoptosis fraction was not proportional to the cell-death rate when the efficacy of anticancer drugs was enhanced with ultrasound (Yu et al. 2004). Indeed, ultrasound lowers the threshold dose for apoptosis and necrosis. Some cells undergo necrosis directly in sonochemotherpy, but undergo apoptosis in conventional chemotherapy. This can be therapeutically applied to deactivate cancer cells with apoptotic malfunction. Ultrasound may cause preliminary damage to cells, thus intensifying drug-induced apoptosis (Fig. 22.2).

Sonochemotherapy has the property of targeted therapy, in that there is a left shift of the dose–anticancer curve and a right shift of the dose–toxicity curve compared with common chemotherapy (Lou et al. 2011). Ultrasound does not necessarily enhance the efficacy of an anticancer agent. There is sometimes no synergism; the efficacy is dependent on the cell type and the drug (Escoffre et al. 2011; He et al. 2012).

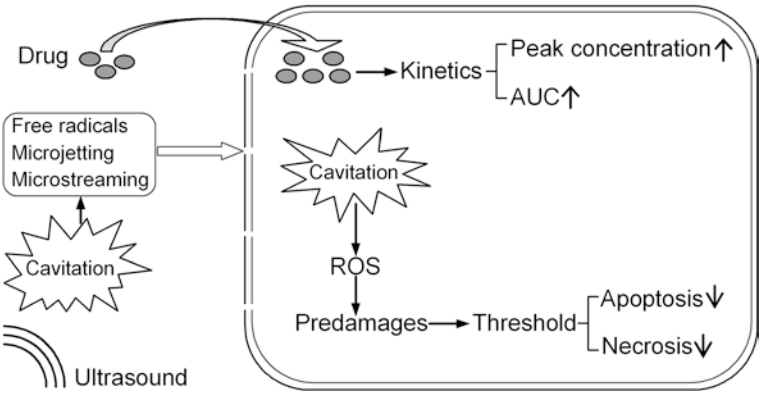

Fig. 22.2 Enhancing the efficacy of an anticancer drug with ultrasound. Cavitation leads to the formation of microjetting, microstreaming, and free radicals, which permeabilize the cell membrane, favoring the influx of drugs. This increases the peak concentration and the area under the concentration–time curve (*AUC*), the pharmacokinetic parameters determining the therapeutic efficacy of a drug. Intracellular cavitation may cause preliminary damage to cells via ROS, thereby decreasing the threshold for apoptosis and necrosis (i.e., ultrasonic chemosensitization)

Ultrasonic cavitation may decrease the potency of a cytotoxic drug (Yu et al. 2011). This should be considered when applying ultrasound to modulate apoptosis in cancer chemotherapy (particularly with high intensities and/or long exposure duration).

22.2.4 Modulating Apoptosis in Other Modalities

The apoptosis rate in SU-DHL-4 cells, attributable to anti-CD20 antibody (rituximab), was increased by 1-MHz insonation; the critical intensities were 0.5 W/cm^2 in air and 2.5 W/cm^2 in CO_2 (Danno et al. 2008). Insonation enhanced apoptosis induced by the combination of tumor-necrosis-factor-related apoptosis-inducing ligand and taurolidine in HT1080 fibrosarcoma cells (Daigeler et al. 2010). Ultrasound enhances the binding of the antibody to the membrane receptor, thereby sensitizing apoptosis. The data indicated that ultrasound can be used to modulate cell death in biotherapy.

Nonlethal insonation sensitized apoptosis attributable to hyperthermia (44 °C). The use of ultrasound (1 MHz, 0.5 W/cm^2) increased the apoptosis rate to 29.9 % in U937 cells, with an enhancement factor of 1.8, and cavitation was considered as the mechanism (Feril et al. 2002). Ultrasound induces preliminary damage, thereby lowering the apoptosis threshold. This makes cells susceptible to hyperthermia.

Ultrasound has been used in molecular therapy for cancers. Apoptosis-related DNA (genes and short hairpin RNA) can be efficiently delivered into the target tissues with insonation, and the efficiency is increased if there are microbubbles (Aoi et al. 2008; Wang et al. 2010). Ultrasound acts as a vector and cavitation is the mechanism in this techniques. Another application is the use of ultrasound to modulate the

expression of a target gene. The therapeutic gene is inserted downstream of a temperature-sensitive promoter. Ultrasound is used to induce a temperature rise, thereby controlling the expression spatially and temporally (Yu et al. 2006).

22.3 High-Intensity Nanosecond Electric Pulses

22.3.1 Electric Effects on Cells

Biomembranes such as plasma, nuclear, and mitochondrial membranes and the endoplasmic reticulum have specific conductivity and dielectric permittivity. The plasma membrane is usually considered as a nonconducting dielectric barrier and a near-ideal capacitor because of its high resistivity under long-duration, slow-rising electric pulses. Indeed, the plasma membrane should be a capacitor connected in parallel with large leaky resistances, especially when fast-rising, ultrashort electric pulses are applied. The cytoplasm and the nucleus are also considered as capacitors in parallel (Hu et al. 2005; Kotnik and Miklavcic 2006).

The applied electric fields should have sufficient magnitude to affect membranes in order to invoke biophysical responses (Joshi and Schoenbach 2010). When external electric fields charge the membrane and induce a sufficiently high potential difference with long enough duration, pores are formed on the membrane, favoring the influx of molecules and ions. These pores reseal within seconds (i.e., reversible electroporation). Reversible electroporation results in an increase of membrane conductivity and redistribution of transmembrane voltage and current (Nuccitelli et al. 2009; Pakhomov et al. 2009). When the transmembrane voltage is higher than 1 V and lasts for more than 2 μs, irreversible electroporation occurs, thereby leading to cell death (Isambert 1998). Nanosecond pulses usually trigger purely electrical responses without thermal effects.

Fast-rising, ultrashort electric pulses induce a nonequilibrium transient and create high values of the potential difference across the inner membranes of the cell. The high potential difference produces localized electric fields across submitochondrial structures, thereby leading to calcium perturbation in mitochondria and the production of forces for the translocation of charged molecules (Vernier et al. 2004; Ren and Beebe 2011). The phosphatidylserine externalization, blockage of action potential, activation of platelets, shrinkage of tumors, and apoptosis induced by high-intensity, ultrashort electric pulses are related to calcium perturbation (Beebe et al. 2002, 2003; Joshi et al. 2007).

High-intensity, ultrashort electric fields can penetrate the plasma membrane and into the inner membranes of organelles. For induction of apoptosis, the amplitude of the electric fields should be high enough and the pulse duration should be nanoseconds or less than nanoseconds. This can be evaluated using the multilayer dielectric sphere model (Fig. 22.3) (Kotnik and Miklavcic 2006). Time domain analysis with the Laplace transformation and the inverse Laplace transformation is used to estimate the voltage across each unit.

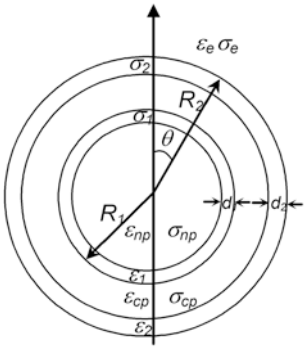

Fig. 22.3 The multilayer dielectric sphere model for a cell in which subcellular structures in the cytoplasm are simplified as a homogeneous unit. R_1 and R_2 are the radius of the nucleus and the cell, respectively, d_1 and d_2 are the thickness of the nuclear envelope and the plasma membrane, respectively, and ε and σ are the dielectric permittivity and conductivity of organelles (*np* nucleus, *1* nuclear envelope, *cp* cytoplasm, *2* plasma membrane, *e* exterior medium)

When cells are subjected to pulsed electric fields (PEFs) with a peak electric strength of E_0, the electrical potential ϕ in each unit can be described as (Kotnik and Miklavcic 2006)

$$\phi_i = \left(A_i r + B_i \Big/ r^2\right) \cos\theta$$

where θ is the angle with respect to the direction of the applied field, and A_i and B_i are coefficients. The electrical potential at $r = 0$ means $B_\mathrm{np} = 0$ and the spatially uniform field at $r \to \infty$ gives $A_\mathrm{e} = -E_0$. The coefficient (A_np, A_1, B_1, A_cp, B_cp, A_2, B_2, or B_e) is determined by the continuity of the electrical potential ϕ and the normal component of the electric current density $\Lambda_i(\mathbf{n}\cdot\nabla\phi)$ at the boundary between two units. **n** is the normal unit vector of spherical coordinates: $\Lambda_i = \sigma_i + \varepsilon_i\partial/\partial\mathrm{t}$ is the admittivity operator of a unit, where $\partial/\partial\mathrm{t}$ is the differential operator transforming a function into its time (t) derivative.

The details of the deduction of these coefficients is beyond the scope of this chapter. The reference values for Jurkat cells in suspension are listed in Table 22.2 (Ermolina et al. 2001; Hu et al. 2005). The voltage of the nuclear plasma (U_np), nuclear envelope (U_1), cytoplasm (U_cp), or plasma membrane (U_2) can be described as follows:

$$\begin{aligned}
U_{np} &= A_{np}\,(t)\,(R_1 - d_1)\cos\theta \\
U_1 &= \left\{A_1\,(t)\,d_1 + B_1\,(\mathrm{t})\left[1 \Big/ R_1^2 - 1 \Big/ (R_1 - d_1)^2\right]\right\}\cos\theta \\
U_\mathrm{cp} &= \left\{A_\mathrm{cp}\,(t)\,(R_2 - d_2 - R_1) + B_\mathrm{cp}\,(\mathrm{t})\left[1 \Big/ (R_2 - d_2)^2 - 1 \Big/ R_1^2\right]\right\}\cos\theta \\
U_2 &= \left\{A_2\,(t)\,d_2 + B_2\,(\mathrm{t})\left[1 \Big/ R_2^2 - 1 \Big/ (R_2 - d_2)^2\right]\right\}\cos\theta
\end{aligned}$$

Table 22.2 Values from the theoretical calculation in Jurkat T cells in suspension

Parameters	Component	Value
Conductivity (S/m)	PM (σ_2)	5.33×10^{-6}
	CP (σ_{cp})	0.13
	NE (σ_1)	4.33×10^{-3}
	NP (σ_{np})	0.18
	EM (σ_{EM})	0.6
Relative permittivity	PM (ε_{r2})	7
	CP (ε_{cp})	60
	NE (ε_{r1})	22.8
	NP (ε_{np})	120
	EM (ε_{EM})	80
Geometry parameters (μm)	Cell radius (R_2)	5.12
	PM radius (d_2)	0.007
	NP radius (R_1)	4.2
	NE thickness (d_1)	0.04

The data were taken from Ermolina et al. (2001) and Hu et al. (2005).
PM plasma membrane, *CP* cytoplasm, *NE* nuclear envelope, *NP* nuclear plasma (nucleus), *EM* external medium

An electric field with a Gaussian (unipolar) or monocyclic (bipolar) shape in the time domain can be generated (Wu et al. 2009). However, no analytical expressions can be obtained when cells in the multilayer dielectric sphere model are exposed to unipolar or bipolar PEFs. These can be approximately described by a sum of ramp functions (Kotnik and Miklavcic 2006). The time-varying Gaussian PEF, $E(t)$, is written as

$$E(t) = E_0 \left[t/T \cdot H(t) - 2\frac{t-T}{T} \cdot H(t-T) + \frac{t-2T}{T} H(t-2T) \right]$$

In the time domain, the PEF can be approximated as a triangular pulse with full width at half maximum, T. The Heaviside function is

$$H(t) = \begin{cases} 1 & t \geq 0 \\ 0 & t < 0 \end{cases}$$

The PEF can be expressed in space of complex frequency with a Laplace transformation:

$$E(s) = E_0 \left[\frac{\left(1 - e^{-sT}\right)^2}{s^2 T} \right]$$

The parameter s refers to the complex frequency, and the transmembrane voltage is a function of s ($\Lambda = \sigma + \varepsilon s$). On the basis of the aforementioned evaluations, the voltage versus time across the nucleus, nuclear envelope, cytoplasm, and plasma

membrane at an anode ($\theta = 180°$) was simulated for electric pulses of 10 μs and 4.8 kV/cm (Fig. 22.4). The data demonstrate the shielding role of the plasma membrane. A PEF with 10-μs duration cannot penetrate into the interior of a cell. The average voltage across the plasma membrane is 1.7 V and the duration is more than 10 μs. Thus, such PEFs lead to irreversible electroporation in the plasma membrane.

Three types of nanosecond PEFs (10, 60, and 300 ns) with equal energy densities were selected and the response voltages of a cell were calculated (Beebe et al. 2003). The peak voltage in the plasma membrane is the highest and that in the cytoplasm is the lowest for a PEF of 300 ns and 26 kV/cm. The potential on the plasma membrane produces nanopores, through which calcium ions flow into the cytosol. The calcium perturbation may invoke calcium-dependent calpain for release of cytochrome *c*. The highest peak voltages occur in the nucleus, the lowest occur in the nuclear envelope, and the voltages in the cytoplasm, on the plasma membrane, and in nuclear envelope are of the same order of magnitude for the PEF of 60 ns and 60 kV/cm. Organelles and membranes, therefore, are the targets. PEFs of 10 ns and 150 kV/cm completely penetrate into the cytoplasm and nucleus, the voltages are mostly distributed in the nuclear plasma and cytoplasm according to the resistances in series, and the lowest voltage is across the membrane (Fig. 22.5). The data show that nanosecond PEFs can penetrate into the cytoplasm and nuclear plasma.

Cytoplasmic voltages induced by nanosecond PEFs can stimulate mitochondrial membranes and endoplasmic reticulum. Electric forces drive the efflux of charged particles such as calcium ions and cytochrome *c*, thus triggerring the physiological responses.

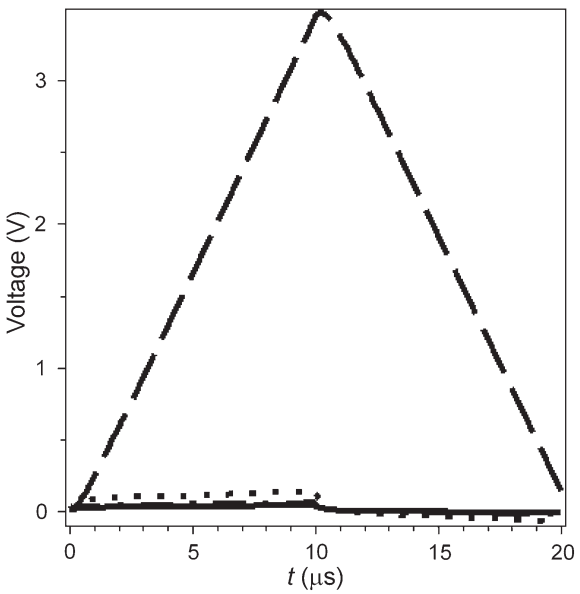

Fig. 22.4 Voltages of each unit with elapsed time for a pulsed electric field (PEF) of 10 μs and 4.8 kV/cm. The data were simulated with the inverse Laplace transformation. *Dashed line* plasma membrane, *dash-dotted line* nuclear envelope, *dotted line* nuclear plasma, *solid line* cytoplasm

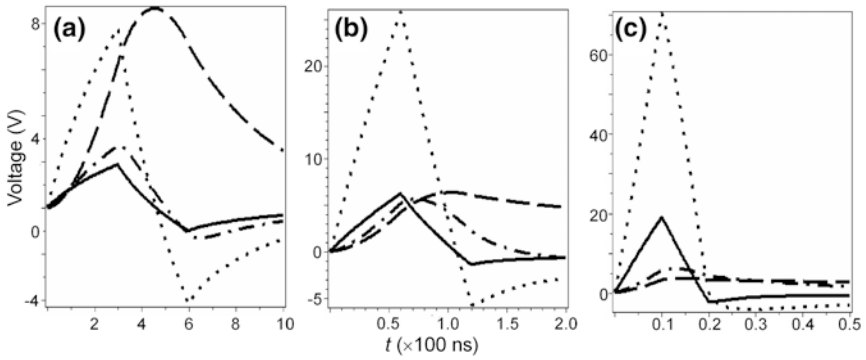

Fig. 22.5 Voltage versus time for PEFs of 300 ns and 26 kV/cm (*a*), 60 ns and 60 kV/cm (*b*), and 10 ns and 150 kV/cm (*c*). The PEF of 300 ns and 26 kV/cm leads to the highest potential on the plasma membrane; the highest voltage occurs in the nucleus for the PEF of 60 ns and 60 kV/cm; the PEF of 10 ns and 150 kV results in higher potential deposition in the nucleus and cytoplasm. The data indicate that a narrow PEF can penetrate through cytoplasmic and nuclear membranes and cause a high potential in the cytoplasm and nucleoplasm, thereby impacting on organelles and DNA directly. *Dashed line* plasma membrane, *dash-dotted line* nuclear envelope, *dotted line* nuclear plasma, *solid line* cytoplasm

22.3.2 Effects on Subcellular Structures

With use of three types of PEFs and reported electric and physical parameters, the effect of PEFs on cytoplasmic organelles was theoretically evaluated from the perspective of release of cytochrome *c* from mitochondria, the initiator of apoptosis (Gowrishankar et al. 2006). A mitochondrion comprises the outer membrane, intermembrane space, inner membrane, and matrix. The parameters for the calculation are summarized in Table 22.3 (Fig. 22.6). The data indicate that a PEF of 60 ns and 60 kV/cm is most effective for the release of cytochrome *c*, which is consistent with experimental findings (Table 22.4) (Beebe et al. 2003). High-intensity pulses exert forces on charged particles, leading to the translocation of these particles (Vernier et al. 2004). Positively charged cytochrome *c* can therefore be driven out along the direction of the electric field (Ott et al. 2002).

22.3.3 Inducing Apoptosis with a High-Intensity Nanosecond PEF

Annexin V staining demonstrated the occurrence of apoptosis, and the rate increased with increasing pulse duration when HeLa cells were subjected to a nanosecond PEF (8–32 ns, 6 kV/cm). The evaluations suggest that subcellular structures may be the target of nanosecond PEFs. Cytochrome *c* in the cytosol was therefore measured. Exposure to a nanosecond PEF led to a higher level of

Table 22.3 Parameters for a mitochondrion

Parameter	Component	Value
Conductivity (S/m)	OM (σ_{OM})	9.5×10^{-7}
	IMS (σ_{IMS})	0.4
	IM (σ_{IM})	47.5×10^{-9}
	MM (σ_{MM})	0.121
	CP (σ_{CP})	0.18
Relative permittivity	OM (ε_{OM})	12.1
	IMS (ε_{IMS})	54
	IM (ε_{IM})	3.4
	MM (ε_{MM})	54
	CP (ε_{CP})	60
Geometry parameters (nm)	Mitochondrion radius (R_M)	300
	OM thickness (d_{OM})	7
	IMS thickness (d_{IMS})	30

The data were taken from Ott et al. (2002).
OM outer membrane, *IMS* intermembrane space, *IM* inner membrane, *MM* matrix, *CP* cytoplasm

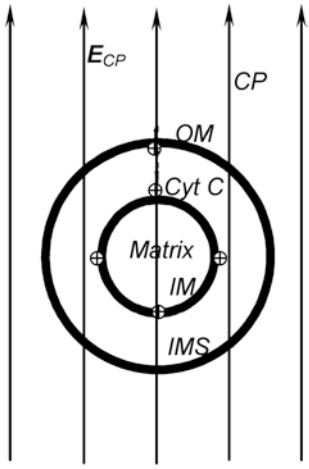

Fig. 22.6 The release of cytochrome *c* (*cyt C*) from mitochondria driven by a PEF. The PEF produces forces that cause positively charged cytochrome *c* to dissociate from the inner membrane (*IM*) and move through the outer membrane (*OM*) along the direction of the electric field.*IMS* intermembrane space, *CP* cytoplasm

cytochrome *c* (Fig. 22.7). The 5-bromo-2′-deoxyuridine incorporation assay confirmed that nanosecond PEFs can induce DNA damage (Fig. 22.8). Electric pulses, therefore, can pass through plasma and nuclear membranes to create sufficiently high voltages in mitochondria and the nucleus without harming the plasma and nuclear membranes (Schoenbach et al. 2007, 2009). Irreparable DNA breakages can lead to cell death via apoptosis (Walker et al. 2006; Ford et al. 2010).

Investigations demonstrated the involvement of the cytochrome *c*–caspase apoptosis pathway in nanosecond PEF treatment and the important role of calcium (Beebe et al. 2003; Ren and Beebe 2011). However, recent data showed that nanosecond PEFs can induce apoptosis via the extrinsic pathway: apoptosis occurred in both $p53^+$ and $p53^-$ cells and there were no reactive oxygen species (Beebe et al.

Table 22.4 Summary of the effects of a pulsed electric field (*PEF*) on the release of cytochrome *c* from mitochondria

	PEF in cytoplasm		
	230 ns, 25.8 kV/cm	54 ns, 86 kV/cm	10 ns, 260 kV/cm
Electric field of IMS (kV/cm)	1	9.5	66.8
Duration of IMS as FWHM (ns)	200	54	7
Favorable for cytochrome *c* detachment/reason	Not good/weak force	Excellent	Bad/shortest time
Average voltage of OM (V)	0.45	0.8	0.89
Duration of voltages across OM (ns)	270	100	35
Permeability of OM/reason	Not good/low U_{OM}	Excellent	Bad/shortest time
Cytochrome *c* release	Moderate	High	Low

OM outer membrane, *IMS* intermembrane space, *FWHM* full width at half maximum

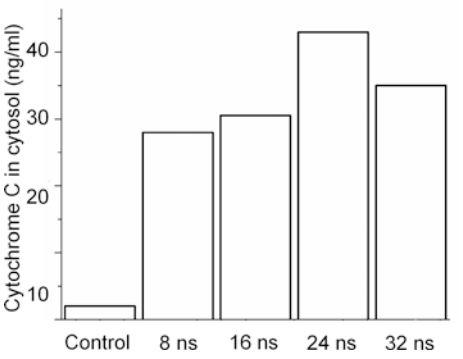

Fig. 22.7 Cytochrome *c* in the cytosol after exposure to nanosecond PEFs. PEFs with a duration of 8, 16, 24, or 32 ns and an intensity of 6 kV/cm led to a higher level, indicating the release of cytochrome *c* from mitochondria

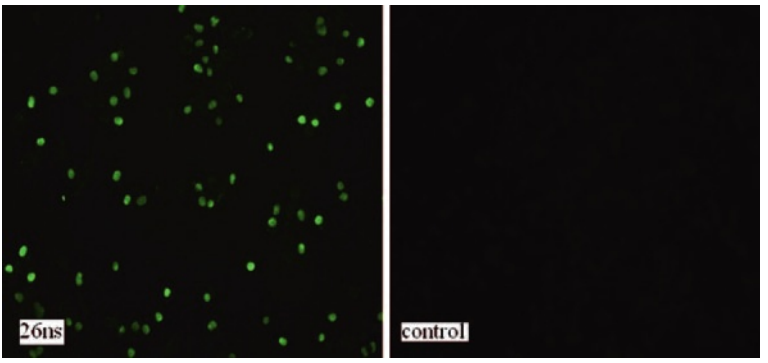

Fig. 22.8 DNA breakages induced by nanosecond PEFs, detected by the 5-bromo-2′-deoxyuridine incorporation assay. Pulses of 26 ns and 6 kV/cm resulted in DNA fractures (*left*), but there was no damage in control cells (*right*)

2002; Hall et al. 2007; Ford et al. 2010). The apoptosis pathway, therefore, may be dependent on the cell type. Electric and experimental data indicate that nanosecond PEFs can directly affect apoptosis modulators such as cytochrome *c*, calpain, Bax, caspase, Bid, and calcium. Theoretical evaluations have revealed that nanosecond PEFs can insult membranes, organelles, and DNA via electrical and mechanical mechanisms, which may result in cell death independent of apoptosis.

A PEF with a duration of more than 100 μs can enhance the efficacy of anticancer drugs (i.e., electrochemotherapy), where PEF facilitated the delivery of drugs into the lesion and drug-induced apoptosis (Dev et al. 2000; Jarm et al. 2010). However, the combination of nanosecond PEFs and cytotoxic agents is not understood and needs to be explored.

22.4 Summary

Ultrasonic cavitation causes oxidative damage to cells, thereby inducing apoptosis. DNA breaks can also be mediated by shear force attributable to cavitation. Use of ultrasound provides a nondrug strategy for enhancing apoptosis in cancer chemotherapy. Cavitation increases membrane permeability, resulting in a higher intracellular drug level. Insonation can alter intrinsic parameters, thus upregulating the cells' sensitivity to a cytotoxic agent. This lowers the threshold dose for both apoptosis and necrosis. The apoptosis pathway in ultrasound-enhanced chemotherapy is usually the same as that in common chemotherapy, depending on the drug applied.

Cellular responses attributable to high-intensity electric pulses are dependent on the voltage applied and the pulse duration. Nanosecond electric pulses pass through plasma and nuclear membranes and produce high voltages in the cytoplasm and the nucleus; this results in apoptosis via the mitochondrial pathway or DNA damage responses. Some apoptosis-related molecules can be modulated with electrical/mechanical mechanisms attributable to electric pulses.

Both ultrasound and electric pulses can be efficiently delivered into predetermined tissues. These two techniques, therefore, can be developed for targeted treatment of cancers.

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Chapter 23
Targeting the Cell Cycle for Cancer Treatment and Neuroprotection

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Abstract Cells traverse the cell cycle through G1 → S → G2 → M phases, and then divide into two daughter cells, which then enter the next cycle or exit to a quiescent G0 phase. This process is tightly controlled by serine–threonine kinases named cyclin-dependent kinases (CDKs). CDKs, as catalytic subunits, become active only in association with their regulatory partner cyclins (e.g., cyclin D–CDK4/CDK6, cyclin E–CDK2, cyclin A–CDK2, cyclin B–CDK1, cyclin C–CDK3). Full activation of the cyclin–CDK holoenzymes requires phosphorylation at particular sites in CDKs. CDK activity is also negatively regulated by direct interaction with CDK inhibitors, which consist of two families, the inhibitor of CDK4 (INK4) family, which specifically inhibit cyclin D-associated kinases, and the kinase inhibitor protein (Cip/Kip) family, which inhibit most CDKs. Dysregulation of these genes (e.g., CDK inhibitors, cyclins, and CDKs themselves) is a common mechanism responsible for out-of-control cell growth, the main characteristic in cancer. Beyond cell cycle regulation, CDKs also play critical roles in gene transcription and neuronal function. In the former case, cyclin T–CDK9 and cyclin C–CDK8 are only involved in transcriptional regulation, whereas cyclin H–CDK7 is involved in regulation of both the cell cycle and transcription. In the latter case, so far CDK5 is the only characterized neuron-specific CDK that appears to function as a double-edged sword dependent on its binding partners (i.e., physiological p35/p39 vs pathological p25). Thus, CDKs are attractive targets for both cancer therapy and neuroprotection, and numerous pharmacological CDK inhibitors have been reported. One major challenge remains whether and how CDK(s) should

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be inhibited in either of the circumstances. This review summarizes current understanding and recent advances in this field.

23.1 Introduction

Cyclin-dependent kinases (CDKs) are a family of serine/threonine kinases that have been implicated in the regulation of cell cycle progression, transcription and neuronal function (Malumbres and Barbacid 2009). So far, about 20 mammalian CDKs have been identified, including CDK1–CDK19 (Malumbres and Barbacid 2005). The activity of most, if not all, CDKs requires the formation of holoenzymes consisting of both CDKs (catalytic subunits) and their partners, in most cases, cyclins (regulatory subunits) (Lapenna and Giordano 2009; Echalier et al. 2010). In words, binding of cyclins (or other partner proteins) is necessary for kinase activity of CDKs. Cyclin binding controls the substrate specificity of CDKs by providing targeting domains, which in turn determine their biological activity. To this end, at least 13 classes (A to L and T) of cyclins have been described so far (Malumbres and Barbacid 2005). Moreover, full activation of CDKs also requires phosphorylation of a threonine residue located between positions 159 and 174 within the T-loop of the kinase domain of CDKs, which is catalyzed by CDK-activating kinase (CAK; composed of CDK7 and cyclin H) (Larochelle et al. 2007). This crucial threonine residue is highly conserved from yeast to mammals. However, cyclin binding is most likely essential for the CAK-mediated phosphorylation of CDKs (Larochelle et al. 2007). Furthermore, the activity of CDKs can also be negatively regulated by phosphorylation of several residues in their active sites. As CDKs play different roles in the cell cycle, transcription, and neuronal function, they can be functionally divided into three classes, i.e., cell-cycle-regulatory, transcriptional, and neuron-specific CDKs.

23.2 Cell-Cycle-Regulatory CDKs

Cell cycle progression provides a mechanism which allows both normal and neoplastic cells to proliferate and grow. The cell cycle is divided into four distinct but tightly related phases, i.e., DNA synthesis (S phase) and mitosis (M phase), which are separated by two gaps (G1 and G2 phases) (Malumbres and Barbacid 2007). Following growth stimuli, cells traverse the cell cycle through G1 $\rightarrow$ S $\rightarrow$ G2 $\rightarrow$ M phases, and then divide to produce two daughter cells, which then enter G1 phase once again to initiate the next cycle or exit from the cell cycle into a quiescent G0 phase (Wesierska-Gadek et al. 2011). The G1 phase contains a transition point referred to as the restriction point which determines whether the cell cycle progression occurs independently of exogenous stimuli (Cicenas and Valius 2011).

Cell cycle progression is tightly controlled by the cyclin–CDK complex composed of cyclin and CDK in a 1:1 ratio. In a number of CDK complexes identified,

CDK1, CDK2, CDK4, CDK6, cyclin A (A1 and A2), cyclin B (B1, B2, and B3), cyclin D (D1, D2, and D3), and cyclin E (E1 and E2) are directly involved in the cell cycle machinery (Canavese et al. 2012). CDKs are the catalytic subunits of the cyclin–CDK complexes, and their activity is regulated by several mechanisms, including their binding to the appropriate cyclin, their folding, and the phosphorylation of a threonine residue in a loop within their structure known as the T-loop (Merrick and Fisher 2012). CDKs are activated by their regulatory partners, members of the cyclin family. Binding of cyclins to this complex induces a conformational change in CDK structure producing a basal, active state (Knockaert et al. 2002). Cyclin–CDK complexes are activated by phosphorylation of CDKs at specific conserved threonine residues (e.g., Thr161 in CDK1, Thr160 in CDK2, Thr172 in CDK4, Thr177 in CDK6) within the T-loop of their kinase domains, a reaction catalyzed by CAK (cyclin H–CDK7 complex) (Larochelle et al. 2007). The activity of CDKs is also regulated by the (de)phosphorylation at conserved tyrosine and threonine residues (Thr14 and Tyr15 in CDK1 and CDK2). These critical residues are phosphorylated by the mixed-lineage kinases Wee1 and Myt1, rendering them inactive (Lapenna and Giordano 2009). The final activation of the cyclin–CDK holoenzymes occurs only after dephosphorylation of these residues, catalyzed by the dual-specificity phosphatases Cdc25s (Cdc25A, Cdc25B, and Cdc25C) (Lents et al. 2002). The activity of these phosphatases is regulated through their protein levels (e.g., Cdc25A) and/or intracellular location (e.g., Cdc25C), which is tightly regulated by proteins of the 14-3-3 family. The 14-3-3 bindings are triggered by phosphorylation of Cdc25s at multiple sites (e.g., Ser178, Thr507, Ser76, Ser123, Ser278, and Ser292 in Cdc25A; Ser309 and Ser361 in Cdc25B; Ser216 in Cdc25C), which in turn lead to βTrCP-dependent degradation via the ubiquitin–proteasome system (e.g., Cdc25A) or sequestration in the cytoplasm (e.g., Cdc25C) (Lapenna and Giordano 2009).

Cyclins are the regulatory components of the cyclin–CDK complexes. Their cellular levels fluctuate through the cell cycle, controlled by a finely tuned balance between de novo synthesis and degradation (Coudreuse and Nurse 2010). Cyclin expression determines a transition specifically from one phase to the next, as well as progression during a particular phase. Cyclins such as cyclins B, A, and E are predominantly regulated by an ubiquitin–proteasome-dependent degradation pathway (Coudreuse and Nurse 2010). These cyclins share a nine-residue sequence in the N-terminal region called the "destruction box," which can be recognized by the enzyme ubiquitin ligase and resulting proteasomal degradation of the cyclins (Lapenna and Giordano 2009). In contrast, D-type cyclins are primarily regulated by transcriptional and translational mechanisms. The perfect timing of individual cyclin expression is controlled by the regulatory elements located in the gene promoters. Cyclins that are no longer needed undergo phosphorylation of specific residues, promoting their recruitment to the Skp1–Cullin–F-box protein or anaphase-promoting complex/cyclosome (APC/C) multiprotein complexes and subsequent degradation through the ubiquitin–proteasome system (Lapenna and Giordano 2009).

CDK activity is also negatively controlled by interactions with endogenous CDK inhibitors, which counterbalance cell cycle progression mediated by cyclin–CDK complexes. The CDK inhibitors are divided two families, the inhibitor

of CDK4 (INK4) family including $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$, which inhibit D-type cyclin-associated kinases (CDK4, CDK6), and the kinase inhibitor protein (Cip/Kip) family containing $p21^{Cip1/Waf1}$, $p27^{Kip1}$, and $p57^{Kip2}$, which efficiently inhibit most CDKs, such as CDK2, CDK4, and CDK6 (Sandal 2002). The regulation of CDK activity by CDK inhibitors is an important mechanism in cell cycle progression after stimulation by mitogenic signals and particularly in tumorigenesis as one or multiple CDK inhibitors are often defected in human cancers. Protein levels of many CDK inhibitors are also regulated through transcriptional (e.g., promoter methylation) and posttranslational (e.g., phosphorylation, ubiquitin–proteasomal degradation) processes.

In mammalian cells, cell cycle progression is regulated by activation of CDKs in sequential order (Ortega et al. 2002) (Fig. 23.1): cyclin D–CDK4/CDK6 holoenzymes promote G1 progression, particularly passing of the restriction point, a point in the G1 phase at which the cell becomes "committed" to the cell cycle, after which extracellular mitogenic stimuli are no longer required. Then, cyclin E–CDK2 complexes act on the G1–S transition, followed by cyclin A–CDK2 on S phase progression. Last, cyclin B–CDK1 complexes control the G2–M transition, mitosis, and M phase exit. Thus, cyclins E, A, and B are expressed during the late G1, S, and G2 phases of the cell cycle, respectively.

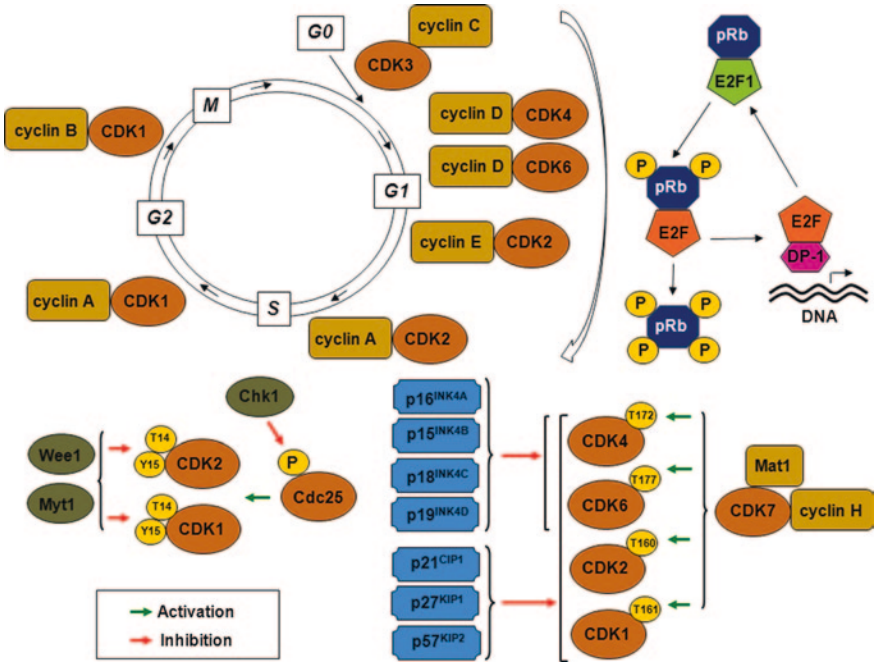

Fig. 23.1 Regulation of the cell cycle by cyclin-dependent kinases (*CDK*). *pRb* retinoblastoma protein

23.2.1 CDK4/CDK6

Cyclin D–CDK4/CDK6 complexes phosphorylate the retinoblastoma tumor suppressor protein (pRb; a primary member of the "pocket protein" family, which also contains p107 and p130), a key negative regulator of cell proliferation (Malumbres and Barbacid 2006). In quiescent cells and early G1 phase, pRb is dephosphorylated or hypophosphorylated, which halts cell cycle progression through interactions with the E2F family of transcription factors and thereby inhibition of their transactivation (Li et al. 2012). After phosphorylation by cyclin D–CDK4/CDK6, pRb is inactivated, releasing E2Fs from an inactive pRb–E2F complex (Yu et al. 2006). E2Fs are thus activated and bind to their heterodimeric partner DP-1, resulting in expression of genes responsible for S phase entry and progression, including cyclin E, which is required for CDK2 activation. Cyclin D–CDK4 can also phosphorylate the other "pocket protein" family members p130 and p107, which may then interact with certain E2Fs (e.g., E2F1 and E2F4) and mimic the function of pRb in *RB1*-null cells (Ciemerych et al. 2008).

Therefore, cyclin D–CDK4/CDK6 complexes are long been believed to be important, perhaps essential components of the core cell cycle apparatus for G1 progress. However, gene knockout of either CDK4 or CDK6, or both, as well as their partner cyclin D is not lethal in mice (Malumbres et al. 2004). Although CDK4 knockout results in insulin-deficient diabetes and partial sterility, mice lacking CDK4 are viable and CDK4$^{-/-}$ mouse embryonic fibroblasts (MEFs) proliferate normally. Moreover, CDK4 and CDK6 double-knockout mouse embryos display normal organogenesis at early stages and most cell types proliferate normally, although they die at late stages because of severe anemia. After serum stimulation, quiescent CDK4$^{-/-}$/CDK6$^{-/-}$ cells are capable of entering S phase. Furthermore, cyclin D1/D2/D3 triple-knockout mouse embryos develop until mid/late gestation and die of heart abnormalities combined with a severe anemia (Kozar et al. 2004). Cyclin D1$^{-/-}$/D2$^{-/-}$/D3$^{-/-}$ MEFs proliferate almost normally but show increased requirement for mitogenic stimulation in cell cycle reentry (Kozar et al. 2004). As a conclusion, cyclin D–CDK4/CDK6 complexes are not as critical for cell cycle progression as previously thought, and these proteins are critically required for proliferation only in selected cell types, such as hematopoietic stem cells.

23.2.2 CDK2

Another important protein kinase involved in interphase progression is CDK2, which targets numerous substrates that are important in DNA replication and gene transcription (Horiuchi et al. 2012). CyclinE–CDK2 and cyclin A–CDK2 regulate the G1–S transition and S phase progression, respectively (Yu and Sicinski 2004). After being phosphorylated by cyclin D–CDK4/CDK6 in late G1 phase, pRb is further phosphorylated by cyclin E–CDK2, leading to complete inactivation of pRb

(Ezhevsky et al. 2001), which is then able to drive the G1–S transition (Merrick et al. 2011). During S phase, cyclin A–CDK2 predominates and phosphorylates various protein substrates involved in DNA synthesis and replication (Wohlbold et al. 2012). Cyclin A–CDK2 also deactivates the E2F proteins, thereby shutting down E2F-dependent transcription (Morris et al. 2002). The active CDK2 complex persists in the nucleus throughout G2 phase. Activation of CDK2 complexes also requires dephosphorylation of Thr14 and Tyr15 by Cdc25s and phosphorylation of Thr160 by CAK (Sandal 2002). CDK2 is phosphorylated at Thr14 and Tyr15 by the dual-specificity kinases Wee1 and Myt1, resulting in inhibition of CDK2 kinase activity. Thr14 and Tyr15 are dephosphorylated by Cdc25s (particularly Cdc25A), leading to activation of both cyclin E–CDK2 and cyclin A–CDK2 (Lapenna and Giordano 2009).

The discovery that mouse embryos can develop normally after knockout of either CDK2 or cyclin E challenges the previous thought that cyclin E–CDK2 activity is strictly required for the cell cycle (Geng et al. 2003). Similarly to the case of cyclin D–CDK4/CDK6, CDK2 knockout mice are viable and develop normally (Ortega et al. 2003). $CDK2^{-/-}$ MEFs proliferate but delay entry into S phase. Quiescent $CDK2^{-/-}$ cells reenter the cell cycle without significant delay in response to stimulation with serum. Therefore, *Cdk2* is not an essential gene in the mouse, although it is required for germ cell development and meiosis (Ortega et al. 2003). On the other hand, cyclin $E1^{-/-}$ and $E2^{-/-}$ mice develop normally, with the exception of deficient spermatogenesis in cyclin $E^{-/-}$ male mice (Yu and Sicinski 2004). Cyclin E1 and cyclin E2 double-knockout embryos die during mid-gestation, caused by placental abnormality (Yu and Sicinski 2004). MEFs from cyclin E-deficient embryos proliferate relatively normally during conditions of continuous cell cycling, but fail to reenter the cell cycle from the quiescent G0 state.

23.2.3 CDK1

Cyclin A–CDK2 and cyclin B–CDK1 govern the G2–M transition (Merrick and Fisher 2010b). The cyclin B–CDK1 complex also regulates the transition of cells into anaphase and through mitosis (Merrick and Fisher 2010a). CDK1 (previously referred to as cdc2) interacts primarily with cyclin B to regulate the G2–M transition (Nurse 2012). Expression of cyclin B is periodic. During interphase, protein levels of cyclin B gradually increase following G1, S and G2 phases, and reach a critical threshold at the end of G2 phase, which activates CDK1, thereby triggering onset of mitosis. During mitosis, cyclin B–CDK1 plays an essential role in control of cell division. The heterodimeric cyclin B–CDK1 complex is also known as maturation-promoting factor, mitosis-promoting factor, or M-phase-promoting factor (MPF) because of its functions in stimulation of the mitotic and meiotic cell cycle. MPF has been shown to execute all the events required to drive cell division (Merrick et al. 2008): (1) after being activated at the end of G2 phase via Thr14 and Tyr15 dephosphorylation by Cdc25, MPF drives the entry into mitosis from G2 phase by phosphorylating multiple proteins required for mitosis; (2)

activated MPF also phosphorylates numerous proteins, such as nuclear lamins (A, B, and C), APC/C, nucleolin, condensins, histones (e.g., H1 and H3), and survivin (Lapenna and Giordano 2009), events critical for cell division. For example, MPF plays an important role in (1) depolymerization of nuclear lamina and breakdown of the nuclear envelope into small vesicles, through phosphorylation-dependent disassembly of the lamins that form an intermediate filament-type network (i.e., nuclear lamina) underlying the inner nuclear membrane; (2) spindle assembly through microtubule instability by targeting various microtubule-associated proteins; (3) chromosome condensation via phosphorylation of condensins; (4) Golgi apparatus and endoplasmic reticulum fragmentation by targeting Golgi matrix proteins such as GM130; and (5) prevention of apoptosis through survivin phosphorylation (Lapenna and Giordano 2009).

Among the substrates of MPF, APC/C drives progression into metaphase by ubiquitinating different regulatory proteins and the resulting proteasomal degradation (Lapenna and Giordano 2009). As the concentration of cyclin B–CDK1 increases, MPF promotes APC/C to polyubiquitinate cyclin B, leading to its degradation and thus disassembling MPF as a negative-feedback loop. Cyclin B degradation by APC/C begins shortly after the onset of anaphase and continues during the period of mitosis, when sister chromatids are separated and pulled toward opposite spindle poles (Lapenna and Giordano 2009).

MPF must be activated and inactivated for the cell to transition from G2 to M phase, and progression and accomplishment of mitosis. Whereas binding of cyclin B is essential for CDK1 activation, activity of MPF is also regulated by phosphorylation and dephosphorylation, as well as subcellular localization (Lapenna and Giordano 2009). Three residues on CDK1 are responsible for the G2–M transition. First, Thr161 must be phosphorylated by CAK (cyclin H–CDK7 complex), which occurs only when cyclin B binds to CDK1. Second, inhibitory phosphorylation of Thr14 and Tyr15 must be removed by Cdc25. During G1 and S phase, MPF is held in the inactive state by Myt1- and Wee1-mediated Thr14 and Tyr15 phosphorylation of CDK1. As Myt1 is a cell-membrane-associated protein kinase, it binds and phosphorylates CDK1 at both Thr14 and Tyr15, thereby sequestering CDK1 in the cytoplasm. Wee1 only phosphorylates Tyr15 and negatively regulates CDK1 activity in the nucleus. At the end of G2 phase, both Myt1 and Wee1 are inactivated (e.g., by MPF, as a positive-feedback loop), whereas a specific dual phosphatase, Cdc25, is activated (e.g., by MPF, representing another positive-feedback loop). Activated Cdc25 then dephosphorylates both Thr14 and Tyr15, activating CDK1 (Lapenna and Giordano 2009). Cdc25C was thought to be responsible for Thr14 and Tyr15 dephosphorylation of CDK1 for the G2–M transition. However, later reports revealed that overexpression of Cdc25A and Cdc25B, but not Cdc25C, may promote activation of CDK1. Furthermore, both Wee1 and Cdc25 are regulated by checkpoint kinase 1 (Chk1) and 14-3-3 through phosphorylation during interphase.

Some CDK complexes, e.g., cyclin A–CDK2 in S phase and cyclin B1–CDK1 in G2/M phase, are associated with the DNA replication competent complex, which may be directly involved in regulation of DNA replication (Hu and

Moscinski 2011). Lastly, cyclin H–CDK7 (known as CAK) activates CDK1, CDK2, CDK4, and CDK6 via phosphorylation at specific threonine residues, events required for full activation of these CDKs.

In the traditional model, cyclin D–CDK4/CDK6 and cyclin E–CDK2 drive cells through interphase via the stepwise phosphorylation of pRb, whereas cyclin B–CDK1 acts primarily only in the G2–M transition. However, later studies revealed that CDK1 is also able to drive the G1–S transition (Santamaria et al. 2007), which challenges the traditional model and suggests that CDK1 may be a pluripotent kinase acting globally throughout the cell cycle (Hu and Moscinski 2011).

As described above, gene knockout of interphase CDKs, including CDK4/CDK6 and CDK2 and their partners cyclin D and cyclin E, is not lethal to mice and their MEFs can still proliferate in a relatively "normal" way (Barriere et al. 2007). These findings raise the possibility that other molecules may compensate for CDK2, CDK4, and CDK6. In $CDK2^{-/-}$ cells, cyclin E binds to CDK1 and forms an active complex, whereas knockdown of CDK1 by short hairpin RNA slows down S phase progression and significantly reduces cell proliferation (Santamaria et al. 2007). CDK2 knockout markedly increases the capability of CDK1 to mediate the G1–S transition (Ortega et al. 2003). Similar phenomena are also found in the case of cyclin D–CDK4/CDK6. In $CDK4^{-/-}$ cells, CDK1 interacts with D-type cyclins (Malumbres et al. 2004). Unlike CDK4/CDK6 and CDK2, CDK1 is essential for early stages of embryonic development. Although knockdown of CDK1 had no effect on interphase progression induced by CDK4 and CDK2 in primary MEFs, CDK1 deletion completely abrogated S phase entry in embryonic cells lacking CDK4/CDK6 and CDK2 (Santamaria et al. 2007). These findings suggesting that CDK1 is a pluripotent CDK that alone is sufficient to drive mammalian cell cycle progression, e.g., promoting entry into S phase as well as the G2–M transition (Hu and Moscinski 2011).

It was thought that only G1 phase CDKs (CDK4/CDK6 and CDK2) phosphorylate pRb. However, both cyclin D–CDK1 and cyclin E–CDK1 are able to phosphorylate pRb proteins in vitro. Actually, whereas inactivation or overexpression of cyclin D–CDK4/CDK6 does not affect pRb activities, increased CDK1 activity might be responsible for pRb phosphorylation (Santamaria et al. 2007). Moreover, CDK1 physically binds to and thus phosphorylates pRb. CDK1 is a target of E2F. In quiescent cells, p130–E2F4 complexes bind to the CDK1 promoter and negatively regulate transcription of CDK1, whereas E2F1, E2F2, and E2F3 bind to positive-regulatory site in the CDK1 promoter and thus induces CDK1 expression. In this context, CDK1–pRb–E2F represents a positive-feedback loop that may amplify CDK1-mediated cell proliferation, whereas inhibition of CDK1 expression may contribute to replication inhibition by pocket protein–E2F complexes.

The CDK inhibitor $p21^{Cip}$ was thought to bind and inhibit cyclin E–CDK2 and/or cyclin D–CDK4, thereby causing G1 arrest. However, $p21^{Cip}$ can directly bind to CDK1. After serum stimulation, both $p21^{Cip}$ and CDK1 locate predominantly to the nucleolus and the levels of the $p21^{Cip}$–CDK1 complexes increase only in $CDK2^{-/-}$ MEFs, but not in wild-type cells (Martin et al. 2005). The

$p21^{Cip}$–CDK1 complex is likely responsible for cell cycle arrest at the G1–S transition in $CDK2^{-/-}$ cells. Moreover, $p21^{Cip}$ is also required for p53-mediated inhibition of CDK1 activity.

Another endogenous CDK inhibitor, $p27^{Kip1}$, was identified as an inhibitor of cyclin E–CDK2 and cyclin D–CDK4. However, $p27^{Kip1}$ can also directly bind to and inhibit CDK1 activity in $CDK2^{-/-}$ MEFs (Martin et al. 2005). Deletion of $p27^{Kip1}$ significantly increases CDK1 activity, which promotes the G1–S transition (Martin et al. 2005). Therefore, $p21^{Cip}$- or $p27^{Kip1}$-induced growth inhibition is, at least in part, due to negative regulation of CDK1 activity.

23.2.4 CDK7

In addition to binding of cyclins, activation of cell-cycle-regulatory CDKs (e.g., CDK1, CDK2, CDK4/CDK6) also requires T-loop phosphorylation. The latter event is catalyzed by CAK (Larochelle et al. 2007). CAK is composed of the catalytic subunit CDK7 and two regulatory subunits, cyclin H and the RING finger protein ménage à trois 1 (Mat1) (Schneider et al. 2002). So far, the trimeric kinase cyclin H–CDK7–Mat1 is the only CAK identified in mammalian cells. CDK7 is activated via binding of cyclin H, whereas its substrate specificity is governed by Mat1 (Schneider et al. 2002).

The phosphorylation by cyclin H–CDK7 is required for activation of cell-cycle-regulatory CDKs in the timing of the transition from one phase to the next as well as progression during individual phases. For example, CDK7 appears to be required for both S phase entry and mitosis in human cancer cells (Wallenfang and Seydoux 2002). Cyclin H–CDK7 phosphorylates cell-cycle-regulatory CDKs at a conserved threonine (Thr161 in CDK1, Thr160 in CDK2, Thr172 in CDK4, and Thr177 in CDK6) located within their T-loop (Larochelle et al. 2007). The activating phosphorylation within the T-loop of CDKs results in a correct structural orientation of amino acids near the active site. This phosphorylation can be reversed by the CDK-associated protein phosphatase KAP, leading to deactivation of CDKs (Larochelle et al. 2007). Unlike in other cell-cycle-regulatory cyclin–CDK complexes, the protein levels of cyclin H and kinase activity of CDK7 do not fluctuate during the cell cycle, suggesting they have other functions beyond cell cycle regulation, such as in gene transcription (see Sect. 23.3).

Later evidence indicated that the absence of CAK activity is completely dispensable for global transcription mediated by RNA polymerase II (RNA pol II) (Ganuza et al. 2012), a well-established target of cyclin H–CDK7–Mat1. However, CDK7 deficiency results in severe mitotic defects in *Caenorhabditis elegans* and *Drosophila melanogaster* without concomitant loss of C-terminal domain (CTD) phosphorylation or transcriptional integrity, respectively (Ganuza et al. 2012). Loss of CDK7 impairs T-loop phosphorylation of cell-cycle-regulatory CDKs (e.g., CDK1 and CDK2), leading to cessation of cell division in vitro and early embryonic lethality in vivo (Ganuza et al. 2012). But it does not affect

transcription mediated by RNA pol II, with the exception of E2F-controlled genes, indicating an indirect consequence of deficient CDK function (Ganuza et al. 2012). As a result, loss of CDK7 expression in adult mice has little effect on non-proliferating tissues, but leads to the premature onset of age-related phenotypes in proliferating tissues, most likely due to depletion of progenitor cells and exhaustion of their renewal capacity. In this context, deficiency of either Mat1 or cyclin H also results in early embryonic lethality in mice (Ganuza et al. 2012).

23.2.5 CDK3

In the concert of the cell cycle, the final missing piece of the puzzle is the regulatory mechanism for the G0–G1 transition, that is, reentry of quiescent G0 cells into the cell cycle. The importance of the G0–G1 transition is underscored by the fact that reentry of commonly quiescent cancer stem cells into the cell cycle is a major reason for recurrence or relapse of cancer after chemotherapy (Sage 2004).

Recent evidence revealing that cyclin C–CDK3 is responsible for the G0–G1 transition (Ren and Rollins 2004) is summarized as follows. In mammalian cells, (1) intracellular levels of cyclin C are high in G0 phase; (2) CDK3, rather than CDK8 (another CDK that is known to bind cyclin C) (Perez et al. 2009; Hoeppner et al. 2005) associates with cyclin C to drive cells from G0 phase to G1 phase by phosphorylation of certain targets, especially pRb (Ren and Rollins 2004); (3) cyclin C–CDK3 phosphorylates pRb at Ser807/811 in G0 phase (Ren and Rollins 2004; Hofmann and Livingston 1996); (4) phosphorylation of pRb by cyclin C–CDK3 is required for cells to exit G0 phase efficiently (Ren and Rollins 2004). Therefore, the G0–G1 transition is regulated through a process similar to the G1–S transition, which is controlled by cyclin E–CDK2, but involves an entirely different cyclin–CDK complex (Sage 2004).

23.2.6 CDK10

CDK10 (previously referred to as PISSLRE) is CDK1-related kinase and has been implicated in the regulation of the G2/M phase of the cell cycle (Kasten and Giordano 2001). CDK10 contains residues that are important regulatory sites in CDK1 and other CDKs, including tyrosine and threonine sites in the ATP-binding domain as well as a threonine residue corresponding to Thr161 of CDK1. As discussed already, the phosphorylation of these sites is critical for activation of CDKs, suggesting that CDK10 may be regulated in a similar fashion. One of CDK10's partners is the transcription factor Ets2. CDK10 binds to the N-terminus of Ets2, thereby inhibiting Ets2 transactivation in mammalian cells (Kasten and Giordano 2001).

23.2.7 CDK14 and CDK15

CDK14 (PFTK1) binds to cyclin Y (Shu et al. 2007). CDK14 is expressed predominantly in mitosis, concurrent with the peak in cyclin Y levels, indicating its role in regulation of mitosis, probably via phosphorylating the substrates in the Wnt pathway.

By analogy, CDK15 (PFTK2) and cyclin Y-like 1 form a similar complex that may share substrate specificity with cyclin Y–CDK14 (Shu et al. 2007).

23.2.8 CDK16

CDK16–CDK18 are very similar and only differ within their N-terminals domains and CTDs. CDK16 can be detected in many tissues (Mikolcevic et al. 2012), particularly in testis and brain. In human cells, CDK16 is phosphorylated at several residues (e.g., Ser119 and Ser153) by protein kinase A (PKA), as well as other residues in the N- and C-terminal extensions (Mikolcevic et al. 2012). Phosphorylation of Ser119 and Ser153 promotes binding of 14-3-3, but the function of these phosphorylations and 14-3-3 binding remains to be defined. CDK16 is activated by membrane-associated cyclin Y, an event inhibited by Ser153 phosphorylation. CDK16 isolated from tissues (e.g., murine testis) is unphosphorylated, interacts with cyclin Y, and exhibits kinase activity (Drexler 1998). Thus, in contrast to other CDKs, the cyclin binding capacity of CDK16 is negatively regulated by phosphorylation. Although CDK16 activity is cell-cycle-related, it is uncertain whether CDK16 itself is involved in regulation of cell cycle progression (Drexler 1998). Interestingly, CDK16 knockout mice develop normally, but male mice are infertile, indicating the essential role of CDK16 in spermatogenesis (Drexler 1998).

23.3 Transcriptional CDKs

Transcription starts with the binding of specific transcription factors to their DNA binding sites in the promoter region of target genes, followed by recruitment of the Mediator complex and multiple general transcription factors (e.g., TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH), and RNA pol II, which together form the preinitiation or pre-elongation complexes (Wesierska-Gadek and Krystof 2009). After the recruitment of RNA pol II to the promoter regions by the general transcription factors, DNA surrounding the transcription start site is melted and allows the transcription initiation and elongation to occur (Wesierska-Gadek and Krystof 2009). After completion of one transcription cycle, RNA pol II is released from DNA. However, several general transcription factors (e.g., TFIIA, TFIID, TFIIE, TFIIH) and the Mediator remain on DNA, forming the scaffold complex, facilitating transcription reinitiation for subsequent cycles of transcription.

The second group of CDKs, functionally different from cell-cycle-regulatory CDKs, consists of kinases involved in the regulation of gene transcription. The well-known transcriptional CDKs are CDK7, CDK8, and CDK9 (Fig. 23.2). These transcriptional CDKs share several features. First, they are subunits of multiprotein transcription-regulatory complexes. CDK7 is a subunit of TFIIH, a general transcription factor component of the preinitiation complex (Glover-Cutter et al. 2009). CDK8 is a part of the CDK module of Mediator (Akoulitchev et al. 2000). CDK9 is the catalytic subunit of positive transcription elongation factor b (P-TEFb), a critical regulator of RNA pol II elongation (Price 2000). Second, they can phosphorylate specific serine residues in the CTD of RNA pol II (Larochelle et al. 2012). CDK7 phosphorylates Ser5 and Ser7, but its major contribution is Ser7 phosphorylation (Glover-Cutter et al. 2009). CDK8 phosphorylates Ser2 and Ser5 within the CTD repeats in vitro (Akoulitchev et al. 2000), but its in vivo contributions remain uncertain. CDK9 is the major Ser2 kinase, but it can also contribute to Ser5 phosphorylation (Larochelle et al. 2012). Third, unlike the cyclin partners of the cell-cycle-regulatory CDKs, the cyclin subunits of transcriptional CDKs—cyclin H for CDK7 (Glover-Cutter et al. (2009), cyclins T1 and T2 for CDK9 (Price 2000), and cyclin C for CDK8 (Barette et al. 2001)—do not exhibit significant oscillations in protein levels during the cell cycle. Last, in addition to cyclin binding, they are also regulated via other interactors, such as repression of

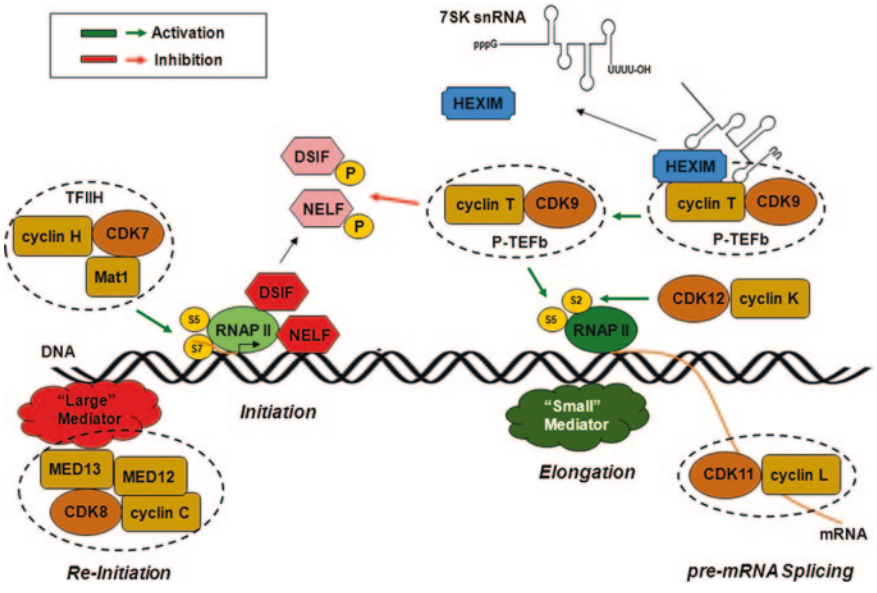

Fig. 23.2 Regulation of gene transcription by CDKs. *DSIF* 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-sensitivity-inducing factor *HEXIM* hexamethylene bisacetamide inducible, *Mat1* ménage à trois 1, *mRNA* messenger RNA, *NELF* negative elongation factor, *P-TEFb* positive transcription elongation factor b, *RNAP II* RNA polymerase II, *snRNA* small nuclear RNA, *TFIIH* transcription factor IIH

CDK9 activity by hexamethylene bisacetamide inducible 1 (HEXIM1), or activation of CDK8 by association with MED12.

23.3.1 CDK7

CDK7 is, so far, the only one atypical CDK that acts at the crossroad between the cell cycle and transcription (Wallenfang and Seydoux 2002; Ganuza et al. 2012). As discussed already, CDK7 plays a key role in cell cycle progression by phosphorylating multiple cell-cycle-regulatory CDKs; therefore, it is known as CAK. On the other hand, the cyclin H–CDK7–Mat1 complex is also a component of the general transcription factor IIH (TFIIH) (Glover-Cutter et al. 2009). The TFIIH holoenzyme consists of the cyclin H–CDK7–Mat1 complex (thus, it is also named TFIIK in this context) and at least six other proteins (XPB, XPD, p62, p55, p44, p34). TFIIK (or CAK) activation is controlled by phosphorylation-dependent binding of the regulatory partners (e.g., cyclin H), and is likely also regulated by other posttranslational modifications. To form a stable complex with its activating partner cyclin H, CDK7 must be phosphorylated at either Ser164 or Thr170 in its own T-loop, which cooperates with binding of Mat1 to stabilize the TFIIK complex. Moreover, binding of Mat1 and Thr170 phosphorylation markedly promotes the activity of CDK7 as a CTD kinase, whereas the substrate specificity of TFIIK for the CTD of RNA pol II requires association with TFIIH.

CTD phosphorylation by cyclin H–CDK7 is responsible for initiation of transcription (Glover-Cutter et al. 2009). The TFIIH complex contains catalytic activities of ATPase, helicase, and kinase, which are required for regulation of different events that control transcription. The helicase activity of TFIIH is involved in ATP-dependent promoter DNA opening, an event required for initiation of transcription. The kinase activity of CDK7 within TFIIH phosphorylates the CTD of RNA pol II, leading to promoter clearance, a step essential for the switch from initiation to elongation during transcription (Larochelle et al. 2012). In this context, the TFIIK complex associates with TFIIH and phosphorylates Ser5 and Ser7 in the CTD of the large subunit of RNA pol II. Ser5 phosphorylation is important for recruitment of chromatin-modifying factors and messenger RNA (mRNA) capping enzymes to the nascent transcript, and Ser7 phosphorylation is specifically involved in regulating small nuclear RNA (snRNA) expression. Moreover, the ability of CDK7 to phosphorylate the CTD is also regulated by phosphorylation of cyclin H. For example, cyclin C–CDK8 phosphorylates cyclin H, resulting in inhibition of the TFIIK activity, and CK2 phosphorylates cyclin H, leading to full activation of TFIIK.

Genetic inactivation of the CDK7 locus revealed that CDK7 is completely unnecessary for global transcription (Ganuza et al. 2012), but is essential for cell cycle regulation (see earlier). Instead, it only affects transcription of a specific cell-division gene cluster, which is less than 5 % of all transcripts (Patel and Simon 2010). Similarly, mouse cells with defective Mat1 also exhibit functional de novo transcription. Thus, CDK7 seems to be only required for the expression of a subset of genes. In addition, the CDK7 complex may also regulate gene expression by

directly phosphorylating transcription factors, including retinoic acid receptor peroxisome-proliferator-activated receptor γ, to either enhance or repress their activity. Therefore, the repertoire of CDK7-responsive genes and the functional requirements for the cyclin H–CDK7–Mat1 complex likely vary in a cell-type-dependent manner.

23.3.2 CDK9

In the elongation phase of transcription, interplay between negative and positive elongation factors regulates the elongation potential of RNA pol II (Canduri et al. 2008). P-TEFb is the first and only known positive elongation factor, and is a complex composed of CDK9 and its partner cyclin T (Price 2000). P-TEFb preferentially phosphorylates Ser2, and most likely Ser5 as well, of the CTD, thereby activating RNA pol II, leading to promotion of transcriptional elongation (Marshall and Price 1995). This event is sensitive to 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), a well-known inhibitor of transcriptional elongation (Garriga and Grana 2004). Moreover, P-TEFb also phosphorylates multiple inhibitory proteins and releases their inhibition on transcription.

Immediately after initiation, transcription is paused via cooperative repression of RNA pol II by two negative elongation factors, DRB-sensitivity-inducing factor (DSIF) and negative elongation factor (NELF) (Peterlin and Price 2006). These factors only bind to the hypophosphorylated form of RNA pol II (IIa), and not to its hyperphosphorylated form (IIo). First, P-TEFb phosphorylates the negative elongation factors DSIF at its SPT5 subunit and NELF at its RD subunit. These phosphorylations release the transcription block of both DSIF and NELF on RNA pol II, permitting the transition into productive elongation (Garriga and Grana 2004). Second, P-TEFb hyperphosphorylates Ser2 of the CTD of RNA pol II that had been previously phosphorylated at Ser5 by CDK7 during transcription initiation. However, although it releases the CTD from the DNA and make it available for CDK9, CDK7-catalyzed Ser5 phosphorylation is most unlikely to be a prerequisite for efficient recognition of Ser2 by CDK9 (Cheng and Price 2007). Ser2 phosphorylation is essential not only for productive transcription elongation, but also for coupling pre-mRNA synthesis with splicing and polyadenylation (Garriga et al. 2010). Last, P-TEFb is recruited to certain promoters by particular transcription factors (e.g., NF-κB and Myc), which may determine the transcription selectivity of target genes.

Like most CDKs, activity of CDK9 relies on binding of its partner cyclin T. Three T-type cyclins have been identified in human cells, named cyclins T1, T2a, and T2b (Peng et al. 1998). The predominant form of human P-TEFb is composed of CDK9 and cyclin T1 (Cheng and Price 2007; Peng et al. 1998). The three T-type cyclins share a highly conserved N-terminus containing an 81 % identical cyclin box. Cyclins T2a and T2b are splice variants of a primary transcript. They share the first 642 amino acids, but cyclin T2b contains a larger CTD that is much less conserved (46 % identity) (Peng et al. 1998). A more distantly related cyclin, cyclin K, may also bind to CDK9 and form an active complex (Fu et al. 1999).

Interestingly, in contrast to T-type cyclin—CDK9 complexes, cyclin K–CDK9 only activates transcription when tethered to RNA rather than DNA. Cyclin K lacks an essential region in its C-terminus, through which cyclin T1 recognizes the CTD of RNA pol II (Fu et al. 1999).

In addition to the known 42-kDa form of CDK9, another isoform (55 kDa) has been identified, and is transcribed from an alternative upstream promoter (Garriga and Grana 2004). The 55-kDa isoform also interacts with cyclin T1, and shares the ability to phosphorylate the CTD of RNA pol II in vitro as well as to stimulate transcription (Garriga and Grana 2004).

Like CDK2, full activation of CDK9 depends not only on cyclin binding but also on phosphorylation of Thr186 within the activation loop (Garriga and Grana 2004). However, unlike CDK2, the Thr186 phosphorylation of CDK9 seems not to be dependent on interaction with cyclin T1. Nevertheless, this phosphorylation causes conformational change in the activation loop, allowing CDK9 to recognize the substrate.

The ratio between the active and inactive forms of P-TEFb is tightly regulated by the actual transcriptional demand in the cell (Van Herreweghe et al. 2007). Cyclin T–CDK9 complexes are inactive when bound to 7SK snRNA and the hexamethylene bisacetamide inducible proteins HEXIM1 (or MAQ1) and/or HEXIM2 (Barboric et al. 2005; Li et al. 2005). The large amount of P-TEFb (e.g., about 50 % in HeLa cells) exists as the kinase-inactive 7SK–HEXIM–P-TEFb complex (termed small nuclear ribonucleoprotein, snRNP), which contains HEXIM1 (and/or HEXIM2) and 7SK snRNA (Barboric et al. 2005; Li et al. 2005). In humans, 7SK RNA is an abundant, RNA polymerase III synthesized snRNA (Yang et al. 2001b). The 7SK snRNA acts as a key regulator of mRNA production by controlling the activity of P-TEFb (Yang et al. 2001b; Nguyen et al. 2001). HEXIM1 and HEXIM2, in homodimeric or heterodimeric form, bind to a distal region of the 5′ hairpin of 7SK snRNA (Li et al. 2005; Michels et al. 2004; Egloff et al. 2006), resulting in a conformational change in HEXIM proteins that enables their C-terminal region to interact with cyclin T1 (Michels et al. 2004). The 3′ hairpin of 7SK snRNA then interacts with cyclin T1, leading to inactivation of P-TEFb (Van Herreweghe et al. 2007; Egloff et al. 2006). In doing so, the 7SK snRNA, in cooperation with the HEXIM proteins, sequesters P-TEFb into the kinase-inactive 7SK–HEXIM1–P-TEFb snRNP, and thus controls the nuclear level of active P-TEFb.

This inhibitory mechanism is abrogated as a result of activation of upstream signaling pathways. For example, inhibition of global transcription by actinomycin D or DRB, or irradiation with ultraviolet (UV) light, triggers rapid disruption of the 7SK–HEXIM–P-TEFb snRNP complex, thereby releasing P-TEFb and increasing the nuclear level of its active form (Krueger et al. 2010). In contrast, inhibition of cell growth can shift the P-TEFb equilibrium toward the 7SK–HEXIM–P-TEFb snRNP complex (Van Herreweghe et al. 2007). Therefore, the nuclear level of active P-TEFb is controlled by dynamic and reversible remodeling of 7SK snRNPs containing RNA helicase A and the heterogeneous nuclear ribonucleoproteins A1, A2/B1, R, and Q (Krueger et al. 2008).

Therefore, activity of the cyclin T–CDK9 complex (P-TEFb) is regulated by at least four mechanisms: (1) the protein levels of its CDK9 and T-type cyclin subunits (Marshall et al. 2005); (2) assembly of cyclin T and CDK9 (Garriga and Grana 2004); (3) inhibition of P-TEFb by 7SK snRNA and HEXIM proteins; and (4) posttranslational modification of P-TEFb components (e.g., Thr186 phosphorylation of CDK9) as well as other regulatory proteins (Fujinaga et al. 2012).

23.3.3 CDK8

CDK8 is a nuclear serine–threonine kinase that functions as a transcriptional regulator. CDK8 is a key component of the Mediator complex (Tsutsui et al. 2011). There are two distinct Mediator complexes for transcriptional regulation, a small complex that activates transcription via RNA pol II, and a large complex that generally represses transcription (Xu and Ji 2011). CDK8 is a subunit of the large Mediator complex (about 1.2 MDa) composed of 25–30 proteins and acts as a molecular bridge between DNA-binding transcription factors and RNA pol II (Fukasawa et al. 2012). CDK8 associates in a dynamic fashion with the Mediator complex as a four-subunit module containing CDK8, cyclin C, MED12, and MED13 (in a 1:1:1:1 stoichiometry, designed the cyclin C–CDK8 module) (Hoeppner et al. 2005). This module is conserved among eukaryotes, and phosphorylates the RNA pol II CTD (Gallorini et al. 2012). MED12 and cyclin C are required for the kinase activity of CDK8 toward the CTD of RNA pol II (Gallorini et al. 2012), whereas MED13 is necessary to recruit the cyclin C–CDK8 module to the small Mediator complex (Fukasawa et al. 2012). In turn, MED13 itself can be phosphorylated by CDK8 (Gallorini et al. 2012). Association of the cyclin C–CDK8 module with the core Mediator enables CDK8 to phosphorylate its substrates such as histone H3 on chromatin. Thus, kinase activity of CDK8 is regulated by association with other subunits of the cyclin C–CDK8 module, whereas its substrate accessibility is regulated via association with the core Mediator.

Cyclin C binds CDK8 and CDK3, which regulate mRNA transcription and the cell cycle, respectively. It is clear that CDK8 acts as a corepressor to negatively affect transcription (Akoulitchev et al. 2000). The inhibitory action of cyclin C–CDK8 is due to disruption of Mediator–RNA pol II interactions likely by MED12 and MED13 (Galbraith et al. 2010), but is independent of the kinase activity of CDK8. The cyclin C–CDK8 module induces conformational change of the core Mediator complex, which physically disrupts the interaction between RNA pol II and the small Mediator complex, thereby blocking the subsequent cycles of transcription (i.e., transcription reinitiation). However, since only a fraction of CDK8 is associated with Mediator, CDK8 may have roles outside this complex. Unlike CDK7 and CDK9, CDK8 prematurely phosphorylates the CTD, thereby preventing, rather than promoting, formation of a transcription initiation complex. In addition, cyclin C–CDK8 phosphorylates cyclin H, repressing CDK7 and thus transcription. Furthermore, CDK8 also phosphorylates certain

gene-specific transcription factors and decreases their stability (Xu and Ji 2011). In summary, CDK8 negatively regulates transcription through at least two distinct mechanisms, i.e., via inhibition of the small Mediator complex, independent of its kinase activity (Galbraith et al. 2010), and as a kinase, by phosphorylating multiple transcription-regulatory proteins. As a consequence, these mechanisms cooperate to negatively control the rate of transcription reinitiation, thereby limiting the quantity of mRNA produced.

However, CDK8 may also play positive roles in transcription in certain circumstances at multiple stages of the transcription cycle (Galbraith et al. 2010). For example, CDK8 has been described as a coactivator in several molecular pathways, including β-catenin-, p53-, and SMAD-mediated signaling pathways, as well as thyroid-hormone-dependent transcription (Galbraith et al. 2010). Therefore, the net influence of CDK8 is determined by both substrate accessibility and gene-specific/stimulus-specific susceptibility. In this context, CDK8 can promote transcription by (1) promoting recruitment of coactivator and transactivation-coupled turnover of transcription factors, (2) cooperating with CDK7 to govern the transition from the preinitiation complex to the scaffold and back to the preinitiation complex during the transcription cycles (Galbraith et al. 2010), (3) facilitating the recruitment of P-TEFb in the elongation phase of transcription, and (4) contributing to chromatin modifications that correlate with transcriptional activation. Several phosphorylation targets of CDK8 have been identified, including the RNA pol II CTD, histone H3, subunits of general transcription factors, and certain transactivators (Galbraith et al. 2010). Thus, the role of CDK8 in transcription is most likely context-specific, dependent on the specific biological contexts and the identity of the transcription factors with which it interacts.

CDK8 knockout in mice is lethal prior to compaction and implantation at embryonic days (Xu and Ji 2011), suggesting a critical role of CDK8 for cell-fate determination in early embryos. Whereas the function and regulation (e.g., their upstream signals) of CDK8 and cyclin C in vivo are still poorly understood, the universal requirement of the CDK8 kinase function in various cellular and developmental contexts and the specific requirements for other conserved module members are unknown.

23.3.4 CDK11

CDK11 is a p110 and p58 PITSLRE protein kinase. Expression of the CDK11p110 isoform is ubiquitous and constant throughout the cell cycle (Trembley et al. 2004). In contrast, CDK11p58 is expressed and functions specifically in G2/M phase (Hu et al. 2003). During apoptosis, a third isoform, CDK11p46, is generated by caspase-dependent cleavage of CDK11p110 and CDK11p58, leaving the catalytic domain intact (Hu et al. 2003).

The CDK11p110-containing complexes influence both transcription and pre-mRNA splicing, suggesting that this CDK may help to link the two processes

(Trembley et al. 2004; Loyer et al. 2005). There are approximately 30,000 genes in the human genome. However, the heterogeneity generated by this number of genes is still not enough to explain the complexity of humans. About 38–74 % of human genes are subject to alternative splicing (Hu et al. 2003), creating more proteomic variations. Moreover, mutations that affect splicing patterns are the underlying causes of some cancers and neurodegenerative diseases. It is estimated that about 15 % of disease-causing mutations in human genes involve misregulation of alternative splicing.

CDK11p110, complexed with cyclins L1 and L2 (Loyer et al. 2008, 2011), belongs to large protein complexes (1-2 MDa and 800 kDa) that contain transcription-related proteins such as RNA pol II, FACT (facilitates chromatin transcription), CK2, and TFIIF (Trembley et al. 2003). The kinase activity of CDK11p110 is functionally required for the regulation of the pre-mRNA splicing process. Several splicing-related factors (e.g., RNPS1 and 9G8) interact with CDK11p110 to regulate transcription and splicing (Loyer et al. 2008). RNPS1 is an SR protein that as a general activator of splicing and a component of the exon–exon junction complex promotes alternative splicing in a substrate-specific manner; 9G8 is another general splicing factor that promotes the nucleocytoplasmic export of mRNA.

23.3.5 CDK12 and CDK13

The functions of CDK12 (CrkRS) and CDK13 (CDC2L5), complexed with cyclins L1 and L2 (Chen et al. 2006, 2007a), were originally related to pre-mRNA splicing (Chen et al. 2006). However, later studies indicated that the endogenous human CDK12 and CDK13 associate with cyclin K, rather than cyclin L (Kohoutek and Blazek 2012). In humans, cyclin K binds CDK12 and CDK13 in two separate complexes (Kohoutek and Blazek 2012). CDK12 and CDK13 are 1,490 and 1,512 amino acid proteins, respectively, both of which contain a conserved central CTD kinase domain (Bartkowiak et al. 2010). Human CDK12 phosphorylate Ser2 in the CTD of RNA pol II in vitro and in vivo (Bartkowiak et al. 2010), whereas CDK13 also phosphorylates the CTD of RNA pol II at least in vitro (Kohoutek and Blazek 2012).

Depletion of cyclin K–CDK12, but not cyclin K–CDK13, results in decreased expression of predominantly long genes with high numbers of exons (Blazek et al. 2011). The most prominent group of downregulated genes are the DNA damage response genes, including *BRCA1* (breast–ovarian cancer type susceptibility protein 1), *ATR* (ataxia telangiectasia and Rad3 related), *FANCI*, and *FANCD2*. Consistent with this, cells that lack cyclin K–CDK12 exhibit spontaneous DNA damage and are sensitive to a variety of DNA damaging agents (Blazek et al. 2011). The essential role of cyclin K–CDK12 is further supported by the fact that genetic inactivation of cyclin K in mice causes early embryonic lethality (Kohoutek and Blazek 2012). In conclusion, cyclin K–CDK12 maintains genomic

stability via regulation of expression of DNA damage response genes. The function of cyclin K–CDK13 is still unknown.

23.3.6 CDK19

CDK19 (previously known as CDK8-like, CDK8L or CDC2L6) (Tsutsui et al. 2011) is also identified in the Mediator complex, and is very similar to CDK8, but is conserved only in vertebrates (Fukasawa et al. 2012). Although CDK19 was sporadically referred to as CDK11 (Tsutsui et al. 2011), it should not be confused with the "splicing kinase" CDK11.

Although CDK8 and CDK19 associate with seemingly identical Mediator complexes (Fukasawa et al. 2012), they are likely not functionally redundant. CDK19 forms the Mediator complexes independent of CDK8. In viral activator VP16-dependent transcriptional regulation, CDK8 supports transcriptional activation, whereas CDK19 represses it (Fukasawa et al. 2012). Both CDK8 and CDK9 bind to same genes regardless of whether they are CDK8's or CDK19's targets, suggesting that Mediator functions as a context-specific transcriptional regulator. CDK8 and CDK19 share the highly conserved kinase and cyclin binding domains, whereas they differ in the C-terminal regions (Tsutsui et al. 2011), which might alter access to substrates or incorporation into complexes. This may provide an explanation for their distinct (likely opposite) functions in regulation of transcription.

23.4 CDKs in Neuron Protection

It is generally believed that neurons are terminally differentiated cells. All CDKs, except CDK5—a neuron-specific CDK, are silenced in postmitotic neurons. However, CDKs appear to be deregulated in several neurodegenerative diseases (Fig. 23.3). Multiple cell-cycle-regulatory CDKs are also related to various pathways required for neuronal death after ischemia/hypoxic injury, particularly stroke. In general, "inappropriate" activation of cell-cycle-regulatory CDKs leads to neuronal death, rather than proliferation, in terminally differentiated (or postmitotic) neurons. Therefore, inhibition of CDKs (e.g., by the pan-CDK inhibitor flavopiridol) is generally neuron-protective. On the other hand, CDK5, complexed with its non-cyclin partner p35 or p39, is the only one postmitotic CDK that functions exclusively in the brain, and plays important functional roles in various aspects of nervous system development and functions, including neuronal migration, neuronal survival, dendritic spine formation, synaptogenesis, adult neurogenesis, neurotransmission, homeostatic plasticity, and learning and memory. In this case, pan-CDK inhibitors such as flavopiridol also inhibit CDK5, which may be harmful to the normal functions of neurons. In contrast, once bound to a smaller but stabler and mislocalized p25 form in some neurodegenerative diseases, CDK5

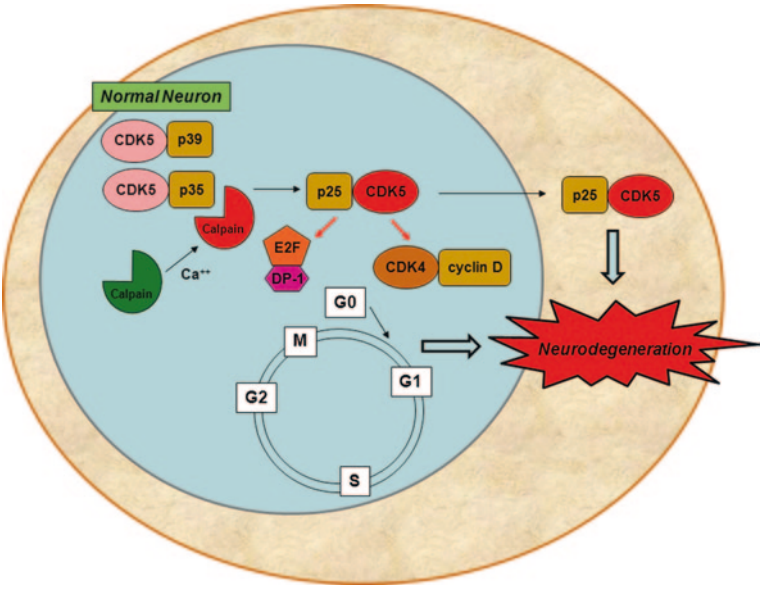

Fig. 23.3 Dysregulation of CDKs in neurodegenerative diseases

becomes a neuron death signal. Thus, it remains uncertain how and which CDKs should be used for the therapeutic purpose of neuron protection in ischemia/hypoxic injury versus neurodegeneration.

23.4.1 CDK5: A Neuron-Specific CDK

CDK5 is a peculiar proline-directed serine–threonine kinase. Unlike other CDKs, CDK5 is not directly involved in regulation of the cell cycle or transcription (Nikolic and Tsai 2000). This kinase is present mainly in postmitotic neurons and its activity is tightly regulated by the interaction with two non-cyclin regulatory components, p35 and p39 (Yamada et al. 2007; Hisanaga and Saito 2003). Kinase activity of CDK5 is mainly determined by the amount of p35 available, which is controlled by a balance between synthesis and degradation (Cruz and Tsai 2004a). CDK5 function is also regulated by phosphorylation, the effect of which effect CDK5 kinase activity is opposite to that of cell-cycle-regulatory CDKs.

CDK5 is a versatile protein kinase that normally regulates multiple neuronal processes such as migration, cortical layering, and synaptic plasticity (Cruz and Tsai 2004a; Hindley and Philpott 2012; Odajima et al. 2011; Cheung and Ip 2007; Cheung et al. 2007). CDK5 also plays an important role in both survival and death of neurons (Hisanaga and Asada 2012; Honma et al. 2003). The pro-survival activity of CDK5 is apparent in neurons when they are exposed to stress

(Cheung and Ip 2004; Cheung et al. 2008), whereas long-term inactivation and/or hyperactivation of CDK5 triggers cell death as seen in neurodegenerative disorders (Hisanaga and Endo 2010). The prodeath activity of CDK5 is suppressed by its membrane association via myristoylation of p35 (Sato et al. 2007; Zhu et al. 2005). Thus, appropriate activity, localization, and regulation of CDK5 is critical for long-term survival of neurons, which is more than 80 years in humans (Asada et al. 2012).

Despite the pivotal role of CDK5 in CNS development, CDK5 dysregulation is significantly implicated in different neuronal diseases, such as Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease, and prion-related encephalopathies (Lopes and Agostinho 2011; Cheung et al. 2006). In these neurodegenerative conditions, CDK5 is overactivated and relocalized owing to association with p25, a truncated form of p35 (Sato et al. 2011; Kamei et al. 2007). The activator switching leads to a shift in the phosphorylation pattern of CDK5 (Cruz et al. 2003, 2006), with an alteration both in target specificity and activity, causing neuronal disorders (Lopes et al. 2007, 2010). For example, in Alzheimer's disease and prion-related encephalopathies, two disorders that share clinical and neuropathological features, CDK5 dysregulation is a linking mechanism between the major neuropathological markers (i.e., amyloid plaques, tau hyperphosphorylation, and synaptic and neuronal loss) (Lopes and Agostinho 2011; Cruz and Tsai 2004b). Moreover, this kinase was shown to be involved in abortive cell cycle reentry (Lopes et al. 2009), a feature recently proposed as a possible step in the neuronal apoptosis mechanism of several neurological diseases (Cruz and Tsai 2004a).

23.4.2 Other CDKs

After death or injury, a neuron, as a terminally differentiated cell, generally cannot be repaired or replaced by neonatal neurons, owing to lack of the capability to grow. In neuronal development and disease, multiple pathways required for neuron death involve various cell cycle molecules (Greene et al. 2007). For example, activation of FOXO1 by CDK1 in cycling cells and postmitotic neurons causes neuronal degeneration (Yuan et al. 2008). On the other hand, microglia become active after injury of the CNS, releasing many types of inflammatory factors to promote neuron apoptosis and aggravate inflammatory injury of tissue; astrocytes proliferate to form a compact glial scar and secret axon regeneration inhibitors, which restrict regeneration of damaged axons and block repair of the structure and recovery of the function of neurons. These pathological processes are closely related to cell cycle regulation. Therefore, cell cycle regulation is related to neuroprotection in two aspects: boosting neuron regeneration and inhibiting activation of microglia and proliferation of astrocytes (Di Giovanni et al. 2005). Appropriate regulation of the cell cycle is an important strategy to protect neurons (Di Giovanni et al. 2005).

Mature neurons mostly rest in G0 phase (Lopes et al. 2009). However, they can reenter the cell cycle in certain pathological conditions such as neurodegeneration and cerebral ischemia (Meijer et al. 1997). The fact that the level of cyclin E is elevated in neurons of patients with Alzhemier's disease suggests that cells progress through S phase (Odajima et al. 2011; Yang et al. 2001a). Likewise, the level of cyclin B1 is increased in hippocampus neurons of patients with vascular dementia, indicating that cells enter G2 phase (Husseman et al. 2000). Further, a modest increase in protein levels of cyclin D1 and CDK4 is observed in brain tissue of cerebral infarcted patients (Love 2003). Commonly, this cell cycle reentry leads to apoptosis, rather than proliferation, of neurons (Liu and Greene 2001b; Wartiovaara et al. 2002). In this context, expression of cyclin D–CDK4, a complex primarily responsible for the G1–S transition, is increased in ischemic cerebral tissue, accompanied by increased apoptosis of neurons (Liu and Greene 2001a; Becker and Bonni 2005). Activation of CDK1, complexed with cyclin B1, is not an indicator of mitosis in neurons. Instead, it prompts neuronal death by direct activation of the proapoptotic protein Bad, before entry to M phase (Konishi et al. 2002). The findings that CDKs are activated in neurons exposed to various apoptotic stimuli further support a notion that activation and expression of CDKs and their partner cyclins may be an important mechanism for neuron apoptosis. In addition to direct involvement in activation of the apoptotic signaling pathways in neurons, neurons may also undergo apoptosis because of abnormally entry to the cell cycle.

In response to injuries, nervous tissue undergoes a series of pathological processes, including ischemia, edema, toxicity of excitatory amino acids, and oxidative stress. However, activation of microglia and proliferation of astrocytes has received attention. Microglia release a number of inflammatory factors, such as TNFα, IL-1β, and IL-6, which play a significant role in initiating secondary injury. On the other hand, astrocytes exhibit marked morphological changes, including thicker neurites and cell hypertrophy, and form glial scar with enhanced expression of intermediate fiber (e.g., glial fibrillary acidic protein) and proliferation of microglia. In the early stage of injury, glial scar is composed of astrocytes, microglia, oligodendrocytes, and infiltrating macrophages; in the later stage, astrocytes substitute other components and become predominant. By quickly replacing damaged tissue, glial scar blocks axonal budding growth and hinders the formation of neural circuits, thus impeding reconstruction and recovery of the nervous tissue. The increase of the levels of cyclins and proliferating cell nuclear antigen (PCNA) in microglia and astrocytes after CNS injury indicates that gliocytes enter the cell cycle and thus undergo division and grow. A strikingly increased number of cyclin D1-, CDK4-, and PCNA-positive cells has been observed in the hippocampal CA1 area after transient total cerebral ischemia, and most of them are microglia and astrocytes (Kato et al. 2003). Granulocyte–macrophage colony-stimulating factor induces activation and proliferation of GMI-M6-3 microglia, in association with elevated levels of cyclins A, D1, and E, as well as a decreased level of $p27^{Kip1}$ (Koguchi et al. 2003). After stimulation with serum, the number of astrocytes isolated from mouse cortex in S phase is increased by 224 % (Di Giovanni et al. 2005). High levels of cyclin D1 and PCNA are also noted in posttraumatic gliocytes. In vitro, astrocytes

are activated and proliferate in response to injury, with a significant increase in 5-bromo-2′-deoxyuridine incorporation (Koguchi et al. 2003). After spinal injury, cyclin A, cyclin B, cyclin E, and PCNA are upregulated, accompanied by increased numbers of OX42/Ki67-positive and glial fibrillary acidic protein/PCNA-positive cells, indicating that gliocytes and astrocytes are activated and proliferate.

In a cerebral ischemia model, after the CDK inhibitor flavopiridol has been administered for 7–9 days after 4 h of global cerebral ischemia/reperfusion, the number of surviving neurons is increased in the hippocampal CA1 area and animal behavior is improved (Koguchi et al. 2003). In a craniocerebral trauma model, the CDK inhibitor roscovitine significantly reduces the number of PCNA-positive cells and the number of cells entering S phase, inhibits cell proliferation, reduces injury, and boosts recovery (Di Giovanni et al. 2005; Meijer et al. 1997). In a spinal injury model, the CDK inhibitor olomoucine prevents expression of cell-cycle-related protein, proliferation of microglia, and release of inflammatory factors, accompanied by reduced glial scarring. It also promotes production of chondroitin sulfate proteoglycan, a secreted factor that regulates cell division (Wartiovaara et al. 2002; Becker and Bonni 2005), neural migration (Konishi et al. 2002; Kato et al. 2003), and axon path finding, as well as stimulating neural stem cell survival. In doing so, olomoucine facilitates reconstruction and recovery (Tian et al. 2006; Tian et al. 2007). Moreover, in an in vitro cutting injury model of astrocytes, olomoucine suppresses cell proliferation and arrest cells in the G1/S and G2/M phases. In a model of photochemically induced ischemia, knockout of cyclin D1 inhibits proliferation of gliocytes, indicating that expression of cyclin D1 is required for division and proliferation of gliocytes (Zhu et al. 2007). In summary, CDK inhibition plays an important role in neuroprotection by at least two distinct but cooperative mechanisms (Di Giovanni et al. 2005): (1) reduction of neural injury and stimulation of neuron replacement and recovery and (2) prevention of inflammatory injury and glial scarring by inhibition of microglia and astrocyte proliferation and inflammatory factor production.

23.5 CDKs in Cancer Treatment

23.5.1 Cell-Cycle-Related Abnormalities in Cancer

Loss of cell cycle control provides a growth advantage to neoplastic cells, thus representing a classic feature of human cancer. Therefore, abnormalities in expression and/or activity of a variety of proteins that directly or indirectly involve the cell-cycle-regulatory machinery play essential roles in the pathogenesis of tumors. These abnormalities usually include loss/inactivation of endogenous CDK inhibitors, overexpression of CDK partner cyclins, amplification/active mutations of CDK genes, or their combination (Malumbres and Barbacid 2009). Such considerations provide a rationale for employing inhibitors of cell cycle progression as anticancer agents.

Aberrations in cell-cycle-regulatory molecules in human cancers occur most frequently in molecules associated with control of the G1 → S transition, a key step which determines initiation of the cell cycle. This signaling pathway is universally disrupted in human cancer even though most human malignancies retain wild-type *RB1* (Li et al. 2012). In fact, dysregulation of the cyclin D–CDK4/CDK6/INK4/pRb/E2F signaling pathway has been identified in more than 80 % of human cancers (Cheung and Ip 2004). In human cancers, the main genetic alterations are deletions (biallelic or monoallelic) or 5′ CpG island methylation of $p16^{INK4a}$ and $p15^{INK4b}$, whereas very few cases or cell lines had $p18^{INK4c}$ or $p19^{INK4d}$ deletions or hypermethylation (Drexler 1998). Among those, the commonest alteration is $p16^{INK4a}$ (CDK inhibitor 2A, a potent tumor suppressor) downregulation because of deletions of gene loci, loss-of-function point mutation, or epigenetic silencing (e.g., by hypermethylation in the promoter region) (Kohno and Yokota 2006; Chakravarti et al. 2007; Auerkari 2006). As a consequence, cancer cells grow uncontrollably owing to hyperactivation of cyclin D–CDK4/CDK6 activity (Yu et al. 2006). For example, whereas deletion of $p16^{INK4a}$ has been found in human primary myeloma cells (Tasaka et al. 1997), a more frequent abnormality in primary myeloma cells is inactivation of $p16^{INK4a}$ and $p15^{INK4b}$ genes by methylation (Chen-Kiang 2003; Ng et al. 1997). 5-CpG island hypermethylation of the $p16^{INK4a}$ locus has been reported in over 50 % of patients with myeloma and related disorders. However, it is uncertain whether hypermethylation of $p16^{INK4a}$ is correlated with a worse prognosis (Lesage et al. 2005; Galm et al. 2004). Inactivation of $p18^{INK4c}$, such as biallelic deletion of $p18^{INK4c}$ and expression of a mutated $p18^{INK4c}$ fragment, is frequently found in myeloma cell lines (Kulkarni et al. 2002). The prognostic significance of such mutations in patients with myeloma is not known. Biallelic deletion of $p18^{INK4c}$ appears to be a late event of myeloma progression (Dib et al. 2006). Hypermethylation or deletion of $p15^{INK4b}$ has been reported in patients with myeloma (Chen-Kiang 2003; Ng et al. 1997), whereas high expression of $p15^{INK4b}$ is associated with diminished proliferative rate and more favorable prognosis in patients with myeloma (Sarasquete et al. 2006). Moreover, concurrent hypermethylation of $p15^{INK4b}$ and $p16^{INK4a}$ has been noted in a significant number of myeloma patients (Chim et al. 2003). Abnormalities in expression of the endogenous CDK inhibitors $p15^{INK4b}$, $p16^{INK4a}$, and $p21^{Cip1/Waf1}$ also often occur in T cell lymphomas (Evens and Gartenhaus 2003).

Overexpression of cyclin D (primarily cyclin D1) is also common in a variety of human cancers (e.g., breast cancer, mantle cell lymphoma, and multiple myeloma) (Li et al. 2012). Aberrant overexpression of cyclin D1 usually stems from gene rearrangement [chromosome inversion or translocations, e.g., t(11p15;q13) and t(11;14)(q13;q32)], gene amplification, or alternative splicing (which generates a cyclin D1b transcript with constitutive nuclear localization and enhanced transforming capacity) (Lu et al. 2003; Carrere et al. 2005; Burd et al. 2006; Krieger et al. 2006; Knudsen et al. 2006). Cyclin D1 is a critical mediator of breast cancer induction by the oncogenes *RAS* and *ERBB2* (Arnold and Papanikolaou 2005). Overexpression of cyclin D (primarily cyclin D1) is also a hallmark of mantle cell lymphoma (Oka et al. 1996). Chromosomal abnormalities are frequently found in

multiple myeloma [e.g., t(11;14)(q13;q32) and t(4:14)(p16;q32)], and often involve cyclin D1 (11q13) (Lesage et al. 2005). Cyclin D1 overexpression is often accompanied by loss of $p16^{INK4a}$, suggesting their possible cooperation in oncogenesis (Shapiro 2006). Overexpression of cyclin D1 results in activation of CDK4/CDK6 owing to an inappropriate increase in the amount of cyclin D–CDK4/CDK6 holoenzyme, and also leads to activation of cyclin E–CDK2 by sequestering Cip/Kip family CDK inhibitors (e.g., $p21^{Cip1}$ and $p27^{Kip1}$) in the cyclin D-dependent kinase complex (Shapiro 2006). Gene amplification and overexpression of cyclins D2 and D3 are also found in some cancers, including B cell malignancies (Delmer et al. 1995; Sonoki et al. 2001). For example, myeloma cells exhibit dysregulation of at least one of the three cyclins [cyclin D1 (11q13), cyclin D2 (MAF/16q23 or MAFB/20q11), or cyclin D3 (6p21)], whereas normal bone marrow plasma cells express low levels of cyclins D2 and D3, and little or no cyclin D1 (Bergsagel et al. 2005). Dysregulation of cyclin D is an early and unifying pathogenic event in myeloma. Patients whose cells exhibit dysregulation of cyclin D1 may have a particularly poor prognosis (Perez-Simon et al. 1998). Cyclin D1–CDK4 and cyclin D2–CDK6 pairing may be a critical determinant for cell cycle reentry and progression in expansion of self-renewing myeloma cells (Ely et al. 2005). Moreover, accumulating evidence also indicates that cyclin D may exert CDK-independent functions (e.g., by acting as a modulator of various transcription factors) in control of cell growth (Tashiro et al. 2007; Fu et al. 2004).

Amplification and point mutations (e.g., $CDK4^{R24C}$ with loss of INK4-binding ability) of the CDK4 gene have also been observed in human cancers (Wolfel et al. 1995). The central mechanism by which dysregulation of the cyclin D–CDK4/CDK6/INK4 pathway contributes to growth advantage of tumor cells involves "unscheduled" inactivation or inhibition of pocket proteins (e.g., pRb, and most likely p107 and p130 as well), resulting in the loss of their function as tumor suppressors. Loss of pRb or hyperactivation of CDK4/CDK6 is found in most human tumor cells (Shapiro 2006; van Deursen 2007). For example, partial or complete deletions in chromosome 13 (e.g., 13q14), which harbors the *RB1* locus, have been reported in up to 30 % of myeloma patients and in up to 70 % of myeloma cell lines. In the remaining cases, pRb is predominantly phosphorylated (Urashima et al. 1996). Abnormalities in chromosome 13 have been associated with a particularly poor prognosis in patients with myeloma (Tricot et al. 1995). Co-expression of CDK4 with oncogenic Ras in normal human epidermal cells induces invasive neoplasia resembling human squamous cell carcinoma (Lazarov et al. 2002). Moreover, it has been found recently that kinase activity of cyclin D1–CDK4 is largely dispensable for normal development, whereas it is critically required for the initiation and maintenance of mammary carcinoma (Landis et al. 2006; Price 2000). Together, cyclin D–CDK4 and cyclin D–CDK6 are a very attractive therapeutic target (Lee and Sicinski 2006; Deshpande et al. 2005).

However, it is noteworthy that the linear model (cyclin D↑ and/or $p16^{INK4a}\downarrow \rightarrow$ CDK4/CDK6↑ → pRb↓) has been challenged. For example, it has been found that the functions of cyclin D–CDK4/CDK6 can be recapitulated by either cyclin D–CDK2 or cyclin E–CDK2 (Horiuchi et al. 2012), both

of which are able to phosphorylate pRb and induce cell proliferation (Barriere et al. 2007). In contrast, loss of CDK2 can also be recapitulated by CDK4, which can phosphorylate pRb even at CDK2-preferred sites, and cyclin E–CDK1 as well (Ortega et al. 2003). This model is further challenged by the recent findings from gene knockout mice (see earlier). In this context, CDK1 is able to replace functions of these interphase CDKs (CDK4/CDK6 and CDK2) by binding to their regulatory partners cyclin D and cyclin E in CDK4/CDK6 or CDK2 knockout mice. Nevertheless, overexpression or constitutive activation of CDK2 has been observed in some types of human cancers (Kohzato et al. 2001; Li et al. 2002; Dong et al. 2001). In addition, overexpression of cyclin A or cyclin E, overexpression/activated mutation of CDK1 or CDK7, and loss of Cip1/Kip family CDK inhibitors (e.g., $p27^{Kip1}$, and most likely $p21^{Cip1}$ as well) have also been reported in many types of human malignancies (Shapiro 2006). Furthermore, loss of endogenous CDK inhibitors (e.g., $p27^{Kip1}$, $p16^{INK4a}$, and possibly $p21^{Cip1}$) is associated with poor outcome in patients with various cancers (Senderowicz and Sausville 2000).

CDK7 and CDK9 phosphorylate the CTD of RNA pol II, facilitating transcription and elongation. The inhibition of this phosphorylation blocks *TP53*-dependent and independent expression of *CDKN1A* (a gene encoding $p21^{Cip1}$). CDK inhibition (e.g., by flavopiridol) stabilizes *TP53*, reducing expression of *MTBP* (mouse TP53 binding protein, transformed 3T3 cell double minute), an important negative regulator of the *TP53* tumor suppressor. Overexpression of *MTBP* is related to several types of human cancer, such as breast cancer, tissue sarcomas, and osteosarcomas (Gallorini et al. 2012).

CDK8 and its regulatory partner cyclin C, two subunits of the Mediator complex, are frequently either mutated or amplified in a variety of human cancers (Xu and Ji 2011). CDK8 functions as an oncoprotein in melanoma and colorectal cancers. CDK8, as one of the most significant colorectal-cancer-associated genes, is the only one that exhibits frequent copy number gain in human colorectal cancers: the CDK8 gene is amplified in 47–76 % of colorectal adenocarcinoma patient samples, and the chromosomal region (13q12.13) that harbors CDK8 is gained in 60 % of colorectal cancers (Gallorini et al. 2012). Thus, CDK8 is a valuable molecular biomarker particularly for the prognosis of a subset of colon cancer patients. The dysregulation of CDK8 is significantly correlated with increased colon-cancer-specific mortality. CDK8 expression is also significantly associated with β-catenin activation in gastric adenocarcinoma (Xu and Ji 2011). Elevated expression of CDK8 predicts poor prognosis in gastric cancers (Firestein et al. 2008).

The gain of CDK8 activity is sufficient to transform 3T3 cells, whereas CDK8 activity is necessary for β-catenin-driven transformation of 3T3 cells. Conversely, knockdown of CDK8 significantly reduces tumor cell growth. CDK8 expression is correlated with high β-catenin activation, expression of tumor suppressor p53, and overexpression of fatty acid synthase (*FASN*), suggesting multiple functions of CDK8 in colorectal tumorigenesis. Indeed, CDK8 is identified as an oncoprotein that promotes the proliferation of colorectal cancer cells (Xu and Ji 2011).

Elevated expression of the CDK8 gene is reported to play a major role in promoting the proliferation of melanoma cells (Xu and Ji 2011), particularly in the subtype of vertical growth phase and metastatic melanomas that display loss of the histone variant macroH2A (Kapoor et al. 2010). Knockdown of either CDK8 or MED12 suppresses the proliferative advantage induced by macroH2A loss in melanoma cells, suggesting that the effect of CDK8 is dependent on the Mediator complex.

However, loss or reduction of expression of CDK8 is also found in a few types of cancers. For example, the CDK8 gene is deleted in esophageal squamous cell carcinoma (Xu and Ji 2011). Likewise, the expression of CDK8 is significantly reduced in bladder cancers. A CDK8 point mutation (D189N) is found in diverse tumor samples, and is likely to cause a loss of CDK8 kinase activity. This implies that CDK8 may not always behave as an oncoprotein in all human cancers, and its activity needs to be tightly regulated.

The *CCNC* gene (encoding cyclin C) is significantly upregulated in patients with gastric cancer, colorectal cancers, adenocarcinoma, leukemia, and lymphoma, as well as a few hepatoma cell lines (Xu and Ji 2011). However, the *CCNC* gene is also frequently deleted in a subset of acute lymphoblastic leukemia, osteosarcoma, and gastric cancers.

CDK10 is an important determinant of resistance to endocrine therapies for breast cancer, whereas CDK10 silencing increases Ets2-driven transcription of c-*RAF*, resulting in mitogen-activated protein kinase pathway activation and loss of tumor cell reliance on estrogen signaling (Iorns et al. 2008). Patients with estrogen-receptor-α-positive tumors that express low levels of CDK10 relapse early when receiving tamoxifen. Low levels of CDK10 are likely associated with methylation of the *CDK10* promoter.

Collectively, these findings strongly support the notion that both cell-cycle-regulatory and transcriptional CDKs are attractive therapeutic targets in human cancer (Firestein et al. 2008).

23.5.2 Pharmacological CDK Inhibitors

CDKs and related molecules are very promising targets in the development of cancer therapeutics (Cicenas and Valius 2011; Canavese et al. 2012; Gallorini et al. 2012; Wesierska-Gadek and Maurer 2011). Among a variety of CDK inhibitors under development and evaluation, several (e.g., flavopiridol, CYC202, UCN-01, and BMS-387032) are currently undergoing clinical evaluation based on preclinical evidence of antitumor activity (Shapiro 2006; Dai and Grant 2004; Schwartz and Shah 2005; Benson et al. 2005). Flavopiridol, as a pan-CDK inhibitor, exerts multiple actions in tumor cells, including inhibition of both cell cycle and transcriptional CDKs (both CDK9 and CDK7), induction of apoptosis, and antiangiogenic activity. UCN-01 was initially developed as a protein kinase C (PKC) inhibitor, and was later found to act as a CDK inhibitor. However, its antitumor effects appear to be more closely related to inhibition of Chk1, leading to “unscheduled” activation

of CDK1 and abrogation of G2/M and S checkpoints, as well as inhibition of the prosurvival 3-phosphoinositide-dependent protein kinase (PDK) 1/Akt pathway. CYC-202 and BMS-387032 have been developed as CDK2 inhibitors, but like most relatively specific inhibitors of CDK2, also inhibit CDK1. In addition, CYC-202 has been also found to inhibit cyclin T–CDK9 and cyclin H–CDK7, thereby blocking phosphorylation of the RNA pol II CTD, which is associated with transcriptional repression of proteins with short half-lives. It is noteworthy that genetic evidence suggests that inhibition of a single CDK (e.g., CDK2) may be insufficient to induce cell death or even prevent cell growth (Tetsu and McCormick 2003), and that inhibition of transcriptional and cell-cycle-regulatory CDKs may cooperate to induce lethality in tumor cells (Cai et al. 2006). Such findings suggest that highly specific CDK inhibitors may be suboptimal as anticancer agents (Merrick and Fisher 2012), and that factors other than or in addition to CDK inhibition very likely contribute to the lethal actions of these compounds (Sausville 2002).

23.5.2.1 Flavopiridol (Alvocidib)

Flavopiridol is a semisynthetic small-molecule derivative of rohitukine, an alkaloid isolated from *Dysoxylum binectariferum* (a plant indigenous to India). In preclinical studies, flavopiridol potently inhibited cell proliferation ($IC_{50} = 66$ nM) in all 60 NCI human tumor cell lines, with no obvious tumor-type selectivity (Senderowicz 2002). As the first clinically relevant CDK inhibitor, initial trials used a schedule of 24 or 72 h continuous infusion every 2 weeks. These schedules achieved concentrations capable of producing preclinical effects. For example, a 72-h infusion regimen produced a 271–415 nM steady-state plasma concentration (Colevas et al. 2002). However, prolonged infusion of flavopiridol proved largely inactive in trials involving several hematopoietic malignancies. Consequently, a bolus administration (1-h infusions for 1–5 days every 21 days) was designed to achieve higher plasma concentrations. Indeed, the 1-h infusion regimen resulted in 1.7–3.8 μM median maximum concentrations, reflecting postinfusion peak concentrations (Colevas et al. 2002), and a limited number of response in certain settings. Notably, clinically achievable concentrations by either continuous or bolus infusion exceeded the threshold for inhibition of CDKs and cell growth, and induction of apoptosis in preclinical studies. However, in striking contrast to its impressive activity in vitro and in various xenograft models (Zhai et al. 2002), the outcomes of most clinical trials were disappointing (Shapiro et al. 2001; Schwartz et al. 2001; Lin et al. 2002; Tan et al. 2002). The failure of flavopiridol to recapitulate its in vitro activity may stem from more than 90 % plasma protein binding and inadequate plasma concentrations of the free drug. In contrast, a variety of clinical trials have demonstrated that combinations of flavopiridol and either conventional chemotherapeutic agents (e.g., paclitaxel, fludarabine, cytosine arabinoside, and irinotecan/CPT-11) or novel signal transduction modulators may be more promising (Karp et al. 2005).

Very recently, a pharmacologically directed infusion schedule has been developed in which half of the flavopiridol dose is administered in 30 min and the other

half in 4 h. This schedule was associated with very promising activity in patients with refractory chronic lymphocytic leukemia(Byrd et al. 2007). In fact, the major dose-limiting toxicity was tumor lysis syndrome. Trials are currently under way to evaluate this schedule in patients with other hematologic malignancies.

Cell Cycle Inhibition

Flavopiridol induces cell cycle arrest by targeting cell-cycle-regulatory CDKs. In in vitro studies using purified CDKs, flavopiridol has been shown to inhibit cyclin B-CDK1 (IC_{50} = 30–40 nM), cyclin A–CDK2 and cyclin E–CDK2 (IC_{50} = 100 nM), cyclin D–CDK4 (IC_{50} = 20–40 nM), cyclin D–CDK6 (IC_{50} = 60 nM), and cyclin H–CDK7 (IC_{50} = 110–300 nM) (Sedlacek 2001; Hardcastle et al. 2002). X-ray crystallographic analysis revealed that L868276 (a deschlorophenyl derivative of flavopiridol with approximately tenfold reduction in inhibitory activity toward CDKs) binds to the ATP-binding pocket of CDK2 (Senderowicz 2002; Hardcastle et al. 2002). In the structure of flavopiridol, the chloro group on the phenyl ring is able to make additional contacts with CDK2, which may explain the tenfold greater potency of flavopiridol compared with L868276. The overall molecular structure of CDKs is quite similar, and they share 40 % sequence homology, including the highly conserved catalytic core region of 300 residues. Flavopiridol directly inhibits the activity of most CDKs by occupying the ATP-binding site of these kinases, an effect that can be competitively blocked by excess ATP. Indeed, CDK1, CDK2, CDK4, and CDK7 in the soluble extracts from non-small-cell lung carcinoma have been shown to bind to immobilized flavopiridol in the absence of ATP but not in its presence. Furthermore, by inhibiting CAK (i.e., cyclin H–CDK7), flavopiridol also prevents phosphorylation at Thr160 and Thr161 of most CDKs (e.g., CDK1, CDK2, CDK4, and CDK6) (Senderowicz 2002), whereas these phosphorylations are necessary for full activation of the CDKs.

Inhibition of CDKs by flavopiridol leads to cell cycle arrest at the G1–S phase transition and the G2–M phase transition, as well as delay in S phase progression (Sedlacek 2001; Shapiro 2004). For example, flavopiridol can block G1 progression by inhibiting cyclin D–CDK4/CDK6, retard S phase progression, or arrest cells in G1 phase by inhibiting cyclin E–CDK2 and cyclin A–CDK2, and arrest cells in G2 phase by inhibiting cyclin A–CDK1 and cyclin B–CDK1. Moreover, flavopiridol also induces cell cycle arrest through transcriptional inhibition and downregulation of cyclin D1, although this action requires slightly higher drug concentrations (100–300 nM) than those necessary for inhibition of cell-cycle-regulatory CDKs (Dai and Grant 2004). It is noteworthy that CDK6 inhibition by flavopiridol seems to play a functional role in cell cycle arrest (e.g., G1 arrest) only in tumor cells lacking CDK4 (Sedlacek 2001). Nevertheless, the patterns of cell cycle arrest (e.g., G1/S arrest, G2/M arrest, or both) induced by flavopiridol (and other pan-CDK inhibitors) appears largely cell-type dependent.

As a first-generation CDK inhibitor, flavopiridol acts as a pan-CDK inhibitor. However, its inhibitory capacity is relatively selective for most CDKs but not for a

specific CDK. Subsequently, efforts have been directed at identifying either structure-based synthetic/semisynthetic compounds or natural products that act on specific CDKs, such as CDK4, CDK1, or CDK2. As a consequence, more specifically selective CDK inhibitors have been developed, for example, inhibitors of CDK4/CDK6 (e.g., PD-0332991 and CINK4) (Hardcastle et al. 2002; Fry et al. 2001) and inhibitors of CDK2 and CDK1 which are significantly less potent against CDK4/CDK6, e.g., CYC202/(*R*)-roscovitine, BMS-387032, PNU-252808, AZ703, NU6102, and NU6140 (Dai and Grant 2003, 2003). A number of these compounds are currently under evaluation as antitumor agents in preclinical models and some are in early stage clinical trials. However, it is difficult to design and develop inhibitors specifically targeting only a single CDK, most likely owing to conservation of amino acids lining the ATP-binding pocket and the high structural homology shared by CDKs. Three-dimensional structural analysis of CDKs, particularly the CDK–inhibitor complex, has provided useful information for the development of novel CDK inhibitors. In particular, the crystal structures of CDK2 have been well established and used extensively for the synthesis of CDK2-specific inhibitors, as well as for evaluating CDK inhibitor potency and selectivity. For example, a new purine-based inhibitor has been described which is 1,000-fold more potent than the parent compound (K_i of 6 nM for CDK2 and 9 nM for CDK1) (Davies et al. 2002). Similar strategies have been used to develop CDK4-specific inhibitors, by using structure-based information related to a CDK4–mimic CDK2 protein (Ikuta et al. 2001; Honma et al. 2001). Moreover, new approaches (e.g., affinity chromatography of immobilized inhibitors) have been established to identify the intracellular targets (selectivity) of individual CDK inhibitors. Clearly, selectivity is a key issue for the use of CDK inhibitors as pharmacological tools to demonstrate the function of CDKs. However, a key question remaining to be answered is whether inhibition of a specific CDK by a highly selective inhibitor, rather than inhibition of broad CDKs by a pan-CDK inhibitor such as flavopiridol, will be efficient in killing tumor cells in view of evidence that (1) tumor cells usually exhibit multiple genetic alterations and/or dysregulation of multiple signaling pathways related to cell cycle regulation and (2) there exists functional overlap and/or cross talk between different CDKs as well as CDKs and other proteins (e.g., their partner cyclins). Thus, it remains possible that the broad actions of a compound such as flavopiridol are beneficial for its antitumor activity.

Transcription Inhibition

Flavopiridol very potently represses transcription (IC_{50} < 10 nM) in vitro by blocking the transition into productive elongation mediated by RNA pol II, which is controlled by P-TEFb (cyclin T–CDK9) (Chao et al. 2000). Flavopiridol inhibits CTD kinase activity of RNA pol II with K_i of 3 nM, a concentration significantly lower than that required for inhibition of most other CDKs (e.g., CDK1, CDK2, and CDK4 with K_i values between 40 and 70 nM). Furthermore, unlike inhibition of other CDKs, inhibition of CDK9 by flavopiridol is noncompetitive with respect to ATP (Chao and Price 2001). A P-TEFb-immobilized assay demonstrates that

flavopiridol (1:1 stoichiometry) remains bound even in the presence of high salt concentrations, suggesting that the apparent lack of competition with ATP could result from very tight binding between the drug and the enzyme. Indeed, recent structural information related to the binary CDK9–flavopiridol complex indicates that flavopiridol binds very tightly to the ATP-binding pocket of CDK9 with higher affinity than CDK2, even though, in contrast to the case of CDK2, no additional binding site has been identified for CDK9 (de Azevedo et al. 2002). In cells, flavopiridol inhibits transcription at concentrations far lower than those required to inhibit CDK1 and CDK2, even in the presence of physiological concentration of ATP (Dai and Grant 2004). Another potential target for transcriptional repression by flavopiridol is CDK7 (catalytic subunit of TFIIH). However, CDK7 inhibition requires higher concentrations of flavopiridol than are necessary for inhibition of CDK9 (Dai and Grant 2003). Therefore, inhibition of transcription by flavopiridol primarily stems from direct inhibition of CDK9.

In mammalian cells, DNA microarrays have shown that flavopiridol inhibits gene expression broadly, similar to the actions of general transcription inhibitors (e.g., actinomycin D and DRB) (Lu et al. 2004). However, at the protein level, flavopiridol primarily downregulates the expression of short-lived proteins, such as cyclin D1 and Mcl-1.

Cyclin D1 Downregulation

Cyclin D1 is a multifunctional protein that not only plays a critical role in regulation of the cell cycle (e.g., the G1–S transition) as a partner of CDK4/CDK6 (see earlier), but also acts as a transcriptional regulator by modulating the activity of several transcription factors (e.g., STAT3) which are CDK-independent, and may explain why cyclin D1 is involved not only in cell cycle progression but also in cell growth and survival (Tashiro et al. 2007; Fu et al. 2004). Recently, it was shown that cyclin D1 binds to the transcription factors STAT3 and NeuroD and inhibits their transcriptional activity, which may be related to modulation of cell differentiation (Coqueret 2002). It has been reported that cyclin D1 also interacts with histone deacetylases, and in doing so blocks access of transcription factors to the promoter and inhibits loading of the initiation complex (Fu et al. 2005). Cyclin D1, as an oncogene, also plays an important role in carcinogenesis, probably by driving cells into S phase and cooperating with various oncogenes (such as Myc and Ras) in malignant transformation (Rodriguez-Bravo et al. 2006). Rearrangement of the cyclin D1 locus and/or overexpression of cyclin D1 have been reported in many human tumors (Hosokawa and Arnold 1998).

Expression of cyclin D1 is growth-factor-dependent, and is regulated at the levels of transcriptional activation, protein degradation, or nuclear export. Mitogen induction of cyclin D1 generally relies on activation of the Ras/Raf/MEK/ERK pathway. Ras signaling and ERK activation promotes transcription of the cyclin D1 gene, probably through transcription factors (Jirmanova et al. 2002). In addition, the Ras signaling pathway is also necessary for associations between

cyclin D1 and CDK4. A variety of transcription factors such as AP-1, STATs (STAT3 and STAT5), NF-κB, Egr-1, Ets, CREB, β-catenin, and certain nuclear receptors activate the cyclin D promoter (Lavoie et al. 1996). On the other hand, expression of cyclin D1 is subject to transcriptional inhibition by other factors, such as E2F1, JunB, INI1/hSNF5, peroxisome-proliferator-activated receptor (nuclear receptor), and calveolin-1 (Hulit et al. 2000). In the control of cell growth rate, the target of rapamycin/eIF4E signaling pathway may act upstream of cyclin D (Shi et al. 2005). The levels of both free and CDK-bound cyclin D1 are also regulated by proteasome-dependent degradation, causing rapid turnover (an approximate half-life of 20 min) of this protein (Diehl et al. 1997). The Ras signaling cascade can prevent ubiquitin–proteasome-dependent degradation of cyclin D1 (Shao et al. 2000). Following its association with CDK4, cyclin D1 is phosphorylated at Thr286 by glycogen synthetase kinase 3β, a kinase controlled by Akt through inhibitory phosphorylation (Takahashi-Yanaga et al. 2006). This event may represent a mechanism by which cyclin D1 is exported from the nucleus to the cytoplasm, resulting in its proteasomal degradation, thereby shutting down this signaling cascade. The pharmacological inactivation of the phosphatidylinositide 3-kinase (PI3K)/Akt pathway and transfection with a constitutively active form of Akt extends the half-life of cyclin D1 twofold to threefold (Radu et al. 2003).

Flavopiridol transcriptionally downregulates expression of cyclin D1 in multiple types of cancer cells. For example, exposure of MCF-7 breast cancer cells to flavopiridol results in a decline in cyclin D1 promoter activity, leading to a decrease in the mRNA and protein levels of cyclin D1 (Carlson et al. 1999). This effect is followed by a decline in the levels of cyclin D3, but not those of cyclin D2 and cyclin E, as well as loss of CDK4/CDK6 activity. In vivo, flavopiridol resulted in depletion of cyclin D1 in the HN12 tumor xenograft, whereas the levels of cyclin D3 and cyclin E remained constant (Patel et al. 1998). Cyclin D1 transcriptional repression may stem from inhibition of P-TEFb by flavopiridol. However, this hypothesis is not supported by observations that inhibition of cyclin D1 expression requires much higher concentrations of flavopiridol (100–1,000 nM) than are required for inhibition of P-TEFb activity. Other mechanisms may involve interruption of transcriptional regulation of cyclin D1 by a number of transcription factors, including positive regulators (e.g., STAT3 and NF-κB) and negative regulators (e.g., E2F1). In addition, flavopiridol can directly bind to duplex DNA with a range of equilibrium dissociation constant values similar to that for the DNA intercalators doxorubicin and pyrazoloacridine (Bible et al. 2000), which may affect the function of DNA as a transcriptional template.

Thus, administration of flavopiridol leads to cell cycle arrest through mechanisms related to inhibition of CDK activities, that is, by direct binding to the ATP-binding sites, by preventing phosphorylation of CDKs through inhibition of CAK (cyclin H–CDK7), or by transcriptional downregulation of cyclin D1. Transcriptional repression of cyclin D1 by flavopiridol may be particularly relevant in mantle cell lymphoma, in which cyclin D1 is overexpressed in 95 % of patients. Notably, flavopiridol has been reported to delay disease progression in a substantial fraction of patients with mantle cell lymphoma (Kouroukis et al. 2003).

Mcl-1 Downregulation

Recent interest has focused on the antiapoptotic protein Mcl-1 as a transcriptional target of flavopiridol. For example, in vitro treatment with flavopiridol induces declines in expression of Mcl-1 mRNA and/or protein levels, which precedes apoptosis, in a variety of cancer cells, including non-small-cell lung cancer cells, multiple myeloma cells, and freshly isolated $CD5^+/CD19^+$ cells from patients with B cell chronic lymphocytic leukemia, and $CD138^+$ cells from patients with multiple myeloma (Pepper et al. 2001; Semenov et al. 2002). Downregulation of Mcl-1 has also been confirmed in vivo in primary leukemic cells from flavopiridol-treated acute myelogenous leukemia (AML) patients (Karp et al. 2005). H1299 (non-small-cell lung cancer) and NIH3T3 (transformed fibroblasts) cells constitutively expressing Mcl-1 are resistant to apoptosis induced by flavopiridol (Ma et al. 2003).

Flavopiridol induces Mcl-1 downregulation most likely by inhibition of P-TEFb (Dai and Grant 2004; Blagosklonny 2004). However, expression of the Mcl-1 gene is controlled by multiple signaling pathways. For example, it is negatively regulated by E2F1 through direct binding to the Mcl-1 promoter, and is positively regulated by the PI3K/Akt pathway, by the mitogen-activated protein kinase pathway, and by transcription factors such as STAT3 and CREB (Wang et al. 1999). Consequently, flavopiridol-mediated downregulation of Mcl-1 may be also related to other mechanisms, including accumulation of E2F1 and disruption of STAT3/DNA binding (Croxton et al. 2002; Lee et al. 2006; Aggarwal et al. 2006).

Apoptosis Induction

In multicellular organisms, cells engage an intrinsic mechanism of self-destruction designated programmed cell death or apoptosis, which is essential for maintaining tissue homeostasis. Tumor cells often have defects in the apoptosis-inducing pathway, resulting in the dysregulated expansion of a population of neoplastic cells, escape of cancer cells from surveillance by the immune system, and resistance to apoptosis induced by chemotherapy and radiotherapy. Initiation of apoptosis involves at least two distinct pathways: the extrinsic pathway, which is mediated by death receptors, and the intrinsic pathway, which is dependent on mitochondria (Strasser et al. 2000). In the former, apoptotic signaling is initiated by binding of members of the TNF family to death receptors, such as CD95 and TNF-related apoptosis-inducing ligand (TRAIL) receptors 1 and 2. When the death receptors are activated by TNF family ligands, their death domains attract the intracellular adaptor protein Fas-associated death domain (FADD), which, in turn, recruits the inactive form of certain initiator caspases (e.g., caspases 8 and 10) to the death-inducing signaling complex (DISC). At the DISC, procaspases 8 and 10 are cleaved and converted into active forms (Fulda and Debatin 2006). In type I cells, the DISC-activated caspase 8 is sufficient to trigger apoptosis directly, but in type II cells, the mitochondria-dependent pathway is required for amplification of initial apoptotic signals, which is linked by truncation/activation of Bid (a proapoptotic

member of the Bcl-2 family) by active caspase 8 (Scaffidi et al. 1998). In the intrinsic pathway, death signals (e.g., DNA damage) lead to mitochondrial damage, probably mediated by caspase 2 activation (Lassus et al. 2002). Mitochondria release cytochrome *c* and other proapoptotic factors, such as AIF and second mitochondria-derived activator of caspase (Smac)/direct inhibitor of apoptosis (IAP) binding protein with low pI (DIABLO), from the intermembrane space to the cytosol, where cytochrome *c* forms a complex (known as the apoptosome) with apoptotic protease-activating factor 1 (APAF1), an inactive form of the initiator caspase (e.g., caspase 9), and ATP (Zou et al. 1999). In the apoptosome, procaspase 9 is cleaved and activated. For both pathways, once the initiator caspases (caspases 8 and 10 in the extrinsic pathway, and caspase 9 in the intrinsic pathway) have been activated, they further cleave and activate executioner caspases (e.g., caspases 3, 6, and 7). Activation of executioner caspases can further result in cleavage/activation of other caspases (including ones lying upstream) to amplify the death-signal cascade (Green and Kroemer 2004). Eventually, the activated executioner caspases cleave a number of cellular "death substrates," leading to biochemical and morphological changes of apoptosis. Apoptotic pathways are tightly controlled by various proteins. For example, processing/activation of caspase 8 is inhibited by a protein referred to as FLIP (FLICE/caspase 8 inhibitory protein) through binding to DISC (Micheau 2003). Importantly, Bcl-2 family members, including antiapoptotic proteins (e.g., Bcl-2, Bcl-xL, Bcl-w, A1, and Mcl-1) and proapoptotic proteins (e.g., multidomain family Bax, Bak, and Bok; BH3-only family Bid, Bim, Bik, Bad, Bmf, Hrk, Noxa, and Puma), play critical roles in regulation of both the intrinsic and extrinsic pathways, primarily at the mitochondrial level (Zamzami and Kroemer 2001). Moreover, IAP protein family members (e.g., XIAP, cIAP1, cIAP2, NAIP, MLAIP, ILP2, livin/KIAP, apollon, and survivin) are antiapoptotic proteins that regulate apoptotic signaling mostly downstream of mitochondria. Most members of the IAP family directly bind to and inhibit the active form of both initiator (e.g., caspase 9) and executioner (e.g., caspases 3, 6, and 7) caspases by promoting their degradation through the ubiquitin–proteasome pathway (Vaux and Silke 2005). In turn, IAPs are inhibited by mitochondria-releasing Smac/DIABLO (Verhagen and Vaux 2002).

It has been well documented that flavopiridol induces apoptosis in a broad spectrum of malignant cells. For example, in vitro, 6–48 h exposure to 100–400 nM flavopiridol induces apoptosis in a variety of tumor cells, including leukemia, lymphoma, head and neck squamous cell carcinoma (HNSCC), breast cancer, non-small-cell lung cancer, prostate carcinoma, gastric carcinoma, esophageal carcinoma, and bladder carcinoma (Shapiro 2004). Human leukemia cells, regardless of their origins (i.e., cultured cell lines or freshly isolated primary cells from patients) or subtype (myeloid, B cell, or T cell type), are the most sensitive to induction of apoptosis by flavopiridol (Kitada et al. 2000). Notably, flavopiridol can also induce apoptosis in tumor cells that are resistant to DNA-damaging agents and radiation (Sedlacek 2001; Shapiro 2004). In vivo, treatment with flavopiridol (5 mg/kg intraperitoneally daily for 5 days) induced apoptosis in the HNSCC xenograft HN12 as detected by terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labeling (TUNEL), with significant reduction (60–70 %) in tumor size (Patel et al. 1998).

The mechanisms by which flavopiridol induces apoptosis have been extensively studied. First, flavopiridol is able to induce apoptosis in tumor cells in which caspase 8 is absent (Achenbach et al. 2000). Moreover, neither the pharmacological caspase 8 inhibitor IETD-FMK nor transfection of the viral caspase 8 inhibitor CrmA is able to block flavopiridol-induced cytochrome *c* release and apoptosis (Decker et al. 2001). These findings suggest that the extrinsic pathway is not primarily involved in flavopiridol-induced apoptosis, despite the fact that cleavage of caspase 8 and Bid has been observed after exposure to flavopiridol (Achenbach et al. 2000).

Flavopiridol induces apoptosis in resting tumor cells which exhibit sensitivities similar to those of proliferating cells, even in the same cell lines (Dai and Grant 2004; Sedlacek 2001), arguing against the possibility that the cytotoxicity of flavopiridol stems from inhibition of CDKs involved in cell cycle regulation. However, direct binding of flavopiridol to duplex DNA may provide an explanation for the ability of flavopiridol to kill noncycling (resting) cancer cells (Bible et al. 2000). Moreover, no significant difference in the cytotoxic activity of flavopiridol has been found between cells expressing pRb versus those defective in pRb expression, even though flavopiridol treatment induces hypophosphorylation of pRb (Cartee et al. 2001). Moreover, certain cell lines that lack detectable pRb expression exhibit more pronounced apoptosis following flavopiridol treatment (Dai et al. 2006). Treatment of H1299 non-small-cell lung cancer cells with flavopiridol (200 nM) results in the rapid elevation of E2F1 levels followed by apoptosis, whereas H1299 cells with deletion of E2F1 through RNA interference or murine embryo fibroblasts deficient in E2F1 are less susceptible but not completely resistant to the cytotoxicity of flavopiridol (Ma et al. 2003). It is known that E2F1 mediates cell death through both p14ARF/MDM2/p53-dependent and p14ARF/MDM2/p53-independent pathways. In most cases, flavopiridol has little or no effect on p53 levels, and its cytotoxic activity appears to be independent of the genetic status of p53 (Reed 2003). There is no direct evidence for the notion that transcriptional downregulation of cyclin D1 contributes to the cytotoxicity of flavopiridol, although repression of cyclin D1 expression by an antisense oligonucleotide approach triggers apoptosis in carcinoma cells (Dai et al. 2006). In contrast, overexpression of cyclin D1 sensitizes human pRb-null myeloma cells (e.g., U266) to flavopiridol (Dai et al. 2006).

Attention has recently focused on transcriptional downregulation of proteins involved in the regulation of apoptosis, which most is likely a central theme underlying the induction of apoptosis by flavopiridol. In this context, Mcl-1 is an important target (see earlier). In addition, flavopiridol also downregulates many other antiapoptotic proteins. For example, administration of flavopiridol results in decreased expression of Bcl-2 in several cell lines, such as B cell leukemia, ovarian carcinoma, prostate carcinoma, and multiple myeloma cells (Semenov et al. 2002). However, flavopiridol-induced apoptosis appears largely independent of Bcl-2 inasmuch as flavopiridol kills tumor cells displaying Bcl-2 overexpression, an event that confers resistance to conventional chemotherapeutic agents (Lavoie et al. 1996). Moreover, neither ectopic overexpression nor antisense-oligonucleotide-mediated downregulation of Bcl-2 affects flavopiridol-induced cell killing (Lavoie et al. 1996). However, human leukemia cells displaying ectopic expression

of N-terminal phosphorylation loop-deleted Bcl-2 (amino acids 32–80, a region known to negatively regulate its function) are highly resistant to flavopiridol-mediated cleavage of Bid, cytochrome *c* release, activation of caspases, degradation of poly(ADP-ribose) polymerase, and apoptosis (Decker et al. 2002), indicating that posttranslational modification(s) (e.g., phosphorylation) of Bcl-2 rather than transcriptional regulation may be involved in flavopiridol-induced apoptosis. Exposure to flavopiridol also results in downregulation of Bcl-xL and XIAP in various types of cancer cells, events likely associated with inhibition of NF-κB (Kim et al. 2003; Takada and Aggarwal 2004). In addition, downregulation of other antiapoptotic molecules (e.g., BAG-1, a regulator of the Hsp70 family that confers resistance to apoptosis induced by a variety of stimuli) has also been reported in B cell chronic lymphocytic leukemia cells exposed to flavopiridol (Kitada et al. 2000).

Other Mechanisms

In several systems, it has been reported that flavopiridol has a significant antiangiogenic activity, which indicates that inhibition of tumor angiogenesis could play a considerable role in the antitumor effects of flavopiridol (Newcomb 2004). The antiangiogenic activity of flavopiridol may be related to the ability of flavopiridol to induce apoptosis in both resting and proliferating endothelial cells through an unknown mechanism which is independent of expression ofCDKs (e.g., CDK1 and CDK2) (Brüsselbach et al. 1998). Indeed, endothelial cells are more sensitive to flavopiridol than other normal cells, such as fibroblasts, bone marrow cells, and peripheral lymphocytes, but are less sensitive than most tumor cells. However, inhibition of vascular endothelial growth factor (VEGF) expression could play an important role in the antiangiogenic effects of flavopiridol. VEGF is an angiogenic factor which is critical for cancer progression and metastasis. In human peripheral blood mononuclear cells and human neuroblastoma cells, it has been shown that flavopiridol completely blocks hypoxia-induced VEGF mRNA transcription and downregulates VEGF protein levels by dramatically decreasing VEGF mRNA stability (Newcomb et al. 2005).

It is also been reported that flavopiridol significantly inhibits rabbit muscle glycogen phosphorylases (a and b) (Oikonomakos et al. 2000). With use of immobilized flavopiridol, glycogen phosphorylases have been identified as flavopiridol-binding proteins (Kaiser et al. 2001). Treatment of A549 non-small-cell lung cancer cells with flavopiridol results in an increase in glycogen accumulation (Kaiser et al. 2001). Further studies showed that flavopiridol inhibits glycogen phosphorylase by directly binding to the inhibitor site in these proteins (Oikonomakos et al. 2000). These findings raise the possibility that interference with glucose homeostasis may also contribute to the antitumor effects of flavopiridol.

23.5.2.2 UCN-01

UCN-01 (7-hydroxystaurosporine, NSC638850, or KW-2401; Kyowa Hakka Kogyo), a derivative of the nonspecific PKC inhibitor staurosporine (a natural

product isolated from *Streptomyces staurosporeus*), was originally developed as a selective PKC inhibitor. It has also been reported to inhibit several CDKs. However, recent studies have shown that UCN-01 exerts other antitumor effects, including inhibition of Chk1, which results in "inappropriate" activation of CDKs and abrogation of DNA-damage-induced cell cycle checkpoints, as well as interference with the PDK1/Akt survival pathway, thus promoting induction of apoptosis. These effects are largely independent of PKC inhibition. UCN-01 displays antitumor activity in in vitro systems and in in vivo xenograft models involving multiple human tumor types, with greater antitumor effects observed with longer administration intervals (e.g., 72 h in in vitro systems). Initial clinical trials of UCN-01 involved a 72-h continuous infusion schedule every 2 weeks (Fuse et al. 2005). Unexpectedly, the plasma half-life (approximately 30 days) of UCN-01 in patients was observed to be 100-fold longer than that observed in preclinical models. It was subsequently shown that UCN-01 extensively binds to plasma α_1-acidic glycoprotein in humans, which accounts for the unique clinical pharmacological behavior of UCN-01 (Dai and Grant 2004; Fuse et al. 2005). On the basis of these findings, further clinical trials are being conducted using modified UCN-01 schedules (e.g., a 36-h continuous infusion every 4 weeks). Such schedules result in a mean UCN-01 half-life of approximately 588 h with peak plasma concentrations of total drug ranging from 30 to 40 μM, with approximately 100 nM concentrations of free UCN-01 detected in saliva (Fuse et al. 2005). Significantly, such concentrations are in excess of those necessary to inhibit Chk1. Several responses have been observed in patients with melanoma and refractory anaplastic large-cell lymphoma. In addition, several phase I trials with shorter schedules (e.g., 3-h infusion) are currently ongoing as combination regimens involving DNA-damaging agents (Dees et al. 2005; Vogel et al. 2007; Sampath et al. 2006).

PKC Inhibition

UCN-01 selectively inhibits Ca^{2+}-dependent PKC isozymes (e.g., PKCα, PKCβ, and PKCγ; IC_{50} = 4–30 nM), and less potently inhibits Ca^{2+}-independent PKC isozymes (IC_{50} ~ 500 nM) (Hofmann 2004). However, it exerts no effect on the atypical PKCs (e.g., PKCδ). In clinical trials, a clear decrease in the level of the phosphorylated cytoskeletal membrane protein adducin, a specific substrate phosphorylated by PKC, was observed in tumor and bone marrow samples following UCN-01 administration (Dai and Grant 2004). However, PKC inhibition appears to be unrelated to various actions of UCN-01, including antiproliferative activity, interference with cell cycle progression, and induction of apoptosis.

CDK Inhibition

UCN-01 can either inhibit or activate CDKs. Crystal structure analysis has shown that UCN-01 binds to active cyclin A–phospho-CDK2 (Johnson et al. 2002). It has been

noted that UCN-01 induces G1 cell cycle arrest at low concentrations (IC_{50} = 100–300 nM) (Akiyama et al. 1997). However, this effect seems unrelated to direct inhibition of CDKs, as UCN-01 inhibits CDK1 and CDK2 in vitro only at higher concentrations (IC_{50} = 300–600 nM). In HNSCC cells, UCN-01 treatment results in G1 block, a phenomenon most likely secondary to depletion of cyclin D3 and induction of the endogenous CDK inhibitors $p21^{Waf1}$ and $p27^{Kip1}$ (Patel et al. 2002). Similar alterations have been observed in HNSCC xenograft (Patel et al. 2002).

Chk1 Inhibition

In normal cells, DNA damage generally induces G1 arrest mediated by accumulation/activation of p53, a major component of the G1 checkpoint machinery (Zhou and Bartek 2004; Bartek and Lukas 2003). In contrast, p53-defective tumor cells primarily arrest in S or G2 phase in the checkpoint response to DNA damage. As most (e.g., more than 50%) human tumors lack p53 function, G2 and S checkpoints play key roles in tumor cell responses to DNA damage. UCN-01 has been found to abrogate the G2 checkpoint selectively in p53-defective cells with 100,000-fold greater (IC_{50} ~ 50 nM) potency compared with caffeine. Chk1 has been defined as a major target in UCN-01-mediated G2 abrogation (Tse et al. 2007; Reinhardt et al. 2007; Vogel et al. 2007). Crystal structure analysis demonstrated that UCN-01 binds the ATP-binding pocket in the Chk1 kinase domain, and the hydroxy group in the lactam moiety of UCN-01 interacts with the ATP-binding pocket, providing a basis for the greater selectivity of UCN-01 toward Chk1 compared with staurosporine and its analogue SB218078 (Zhao et al. 2002).

Pharmacological concentrations of UCN-01 inhibit the activity of both Chk1 and checkpoint kinase 2 (Chk2) immunoprecipitated from human tumor cells, which may account for the observation that UCN-01 abrogates IR-induced p53-independent G2 arrest, whereas Chk1 activity remains unchanged (Yu et al. 2002b). UCN-01 was also shown to block Cdc25C phosphorylation mediated by another kinase, C-TAK1, which inhibits Cdc25C constitutively in the absence of DNA damage (Karlsson-Rosenthal and Millar 2006; Kohn et al. 2002). Therefore, regardless of which kinase is responsible for the phosphorylation/inactivation of Cdc25C, inhibition of this event by UCN-01 results in “inappropriate activation” of CDK1 that drives tumor cells through mitosis prior to repair of DNA damage, resulting in apoptosis (Callegari and Kelly 2007; Harrison and Haber 2006). Plasma samples isolated from patients who received UCN-01 were found to induce 40–70 % abrogation in an ex vivo G2 checkpoint assay (Kawabe 2004).

UCN-01 has also been reported to abrogate the S phase checkpoint (Gottifredi and Prives 2005), but the mechanisms responsible for this event appear to be complex. In p53 mutant tumor cells, low concentrations of UCN-01 causes S phase cells to progress to G2 phase before undergoing mitosis and cell death, whereas high concentrations (approximately 500 nM) lead to rapid premature mitosis and death of S phase cells. The latter event may stem from rapid Cdc25C activation by C-TAK1 inhibition (Kohn et al. 2002). IR-induced S checkpoint response can be divided into fast (less than 2 h) and slow (more than 1–6 h) processes. The ataxia

telangiectasia mutated (ATM)-dependent pathway controls only the fast response, whereas the slow response is controlled by an ATM-independent pathway involving Chk1 (Dai and Grant 2004). These results are consistent with observations that UCN-01 abolishes the UV-induced S checkpoint response through inhibition of ATR-dependent Chk1 activation (Heffernan et al. 2002).

These findings have created a theoretical basis for developing a therapeutic strategy in which UCN-01 may sensitize tumor cells (particularly p53-defective cells) to DNA-damaging agents and radiation by abrogating the G2 and/or S checkpoints. It is noteworthy that the checkpoint abrogation effects of UCN-01 are manifested at lower drug concentrations (e.g., IC_{50} ~ 50 nM for G2 checkpoint abrogation) than those responsible for cytotoxicity or inhibition of cell proliferation.

PDK1/Akt Inhibition

The PI3K/Akt cascade is a critical signaling pathway in cell survival mediated by many growth factors and cytokines. Phosphorylation of Akt at Thr308 is catalyzed by PDK1 and phosphorylation at Ser473 is catalyzed by PDK2. UCN-01 directly inhibits upstream Akt kinase PDK1 with IC_{50} < 33 nM in in vitro and in vivo assays, whereas enforced expression of PDK1 restores Akt kinase activity (Sato et al. 2002). Crystal structure analysis demonstrated that UCN-01 binds to the kinase domain of PDK1 more specifically than staurosporine (Komander et al. 2003). Overexpression of active Akt diminishes the cytotoxic effects of UCN-01, indicating that inhibition of the PDK1/Akt pathway is attributed to the antitumor activity of this agent (Sato et al. 2002).

Apoptosis Induction

UCN-01 induces apoptosis with IC_{50} values of 100–1,000 nM in a panel of HNSCC cell lines in vitro and in HN12 xenograft in vivo, and exhibits enhanced cytotoxicity in cells displaying mutant p53 (Patel et al. 2002). Although the mechanism underlying UCN-01-induced apoptosis is still unknown, several potential targets have been postulated. First, inhibition of PDK1/Akt has been directly related to the cytotoxicity of UCN-01 (Sato et al. 2002). Second, as CDK1 is identified as a proapoptotic mediator (Castedo et al. 2002), Chk1/Chk2–Cdc25C-mediated CDK1 activation, particularly under "inappropriate" circumstances, may contribute to induction of apoptosis by UCN-01 (see earlier). Finally, UCN-01-induced apoptosis has been associated with downregulation of antiapoptotic proteins, such as Mcl-1, XIAP, BAG-1, and Bcl-2 (Zhao et al. 2002).

23.5.2.3 CYC202

CYC202 [(*R*)-roscovitine, seliciclib; Cyclacel] is a substituted purine analogue derived from 6-dimethylaminopurine and isopentenyladenine. In vitro kinase

assays using purified recombinant kinases have revealed that CYC202 inhibits CDK2 (cyclin E–CDK2, IC_{50} = 100 nM; cyclin H–CDK7, IC_{50} = 490 nM; cyclin A–CDK2, IC_{50} = 710 nM), and less potently CDK1 (cyclin B–CDK1, IC_{50} = 2.69 μM), but neither CDK4 (cyclin D1–CDK4, IC_{50} = 14.21 μM) nor other kinases (e.g., PKA and PKC) (Benson et al. 2005; McClue et al. 2002). Like most CDK inhibitors, CYC202 inhibits CDKs by competing with ATP for its CDK binding site (Tang et al. 2005; Bach et al. 2005). In vitro evaluation of antitumor activity demonstrated the cytotoxicity of CYC202 (average IC_{50} = 15.2 μM) against a panel of 19 human tumor cell lines, including those with cisplatin- and doxorubincin-resistant phenotypes, independent of p53 status and cell cycle alterations (McClue et al. 2002). In vivo administration of CYC202 resulted in a significant antitumor effect and reduction in tumor growth rate in mouse xenograft bearing human colorectal carcinoma and uterine cancer (McClue et al. 2002; Raynaud et al. 2005). On the basis of these findings, CYC202, the first oral bioavailable CDK inhibitor, has entered phase I clinical trials in patients with advanced solid tumors (Benson et al. 2007). These studies revealed that maximum plasma concentrations of more than 2,000 ng/ml at day 1 and day 7 were achievable at 800 mg/kg twice a day for 7 days, a dose in the range of the IC_{50} values reported for seliciclib in vitro activity and without dose-limiting toxicity (Benson et al. 2007).

The anticancer activity of CYC202 has been related to (1) inhibition of cell-cycle-regulatory CDKs (e.g., CDK1, CDK2, and CDK7) and downregulation of cyclin D1, leading to a reduction in pRb phosphorylation at multiple sites and cell cycle arrest in G1, S, and G2–M phases (Whittaker et al. 2004; Lacrima et al. 2005, 2007); (2) inhibition of transcriptional CDKs (e.g., CDK9 in particular, and CDK7), resulting in decrease/inactivation of RNA pol II and transcriptional repression of short-lifetime proteins such as cyclin D1, cyclin A, cyclin B1, as well as Mcl-1 and XIAP (Whittaker et al. 2004; Lacrima et al. 2005; Hahntow et al. 2004); (3) more importantly, induction of apoptosis in tumor cells while largely sparing normal cells (Alvi et al. 2005), which is most likely related to downregulation of antiapoptotic proteins, particularly Mcl-1 (Zhang et al. 2002; Maccallum et al. 2005; Rossi et al. 2006); and (4) lowering the threshold of cancer cells to cytotoxic agents or other novel agents (Maccallum et al. 2005; Coley et al. 2007a, b; Ribas et al. 2006).

23.5.2.4 BMS-387032 (SNS-032)

A series of compounds derived from 2-acetamidothiazolythioacetic ester have been discovered and optimized as small-molecule inhibitors of cyclin E–CDK2. Among these, BMS-387032 has been identified as an oral bioavailable CDK2 inhibitor. This compound selectively inhibits CDK2 (IC_{50} = 48 nM, 10-fold and 20-fold selective over cyclin B–CDK1 and cyclin D–CDK4, respectively) (Misra et al. 2004). X-ray crystallographic analysis demonstrated that these compounds bind to the active ATP-binding site of the CDK2 protein. BMS-387032 displays

marked antiproliferative activity, with $IC_{50} = 95$ nM in A2780 ovarian carcinoma cells (Misra et al. 2004). Similar effects were observed in a panel of tumor cell lines in vitro. BMS-387032 has demonstrated significant antitumor activity in vivo in both a murine tumor model and human tumor xenograft models (Misra et al. 2004). This systematic investigation led to a phase I clinical trial of BMS-387032 (Senderowicz 2003). Initial results from the clinical trial showed some objective tumor responses and good tolerability.

These effects in all likelihood stem from rapid induction of apoptosis and cell cycle arrest (Dai and Grant 2004; Senderowicz 2003). BMS-387032 induces E2F1 but diminishes E2F4 levels, whereas E2F1-deficient fibroblasts are less sensitive to this agent (Ma et al. 2004). A similar phenomenon has been observed in human breast cancer cells, in which treatment with BMS-387032 leads to stabilization of E2F1 (Ma and Cress 2007). Moreover, this event is accompanied by a significant increase in the p57 mRNA and protein levels, whereas p57-deficient cells are more sensitive to BMS-387032-induced apoptosis, indicating that this event may serve to limit E2F1-mediated cell death. In human lung carcinoma cells, BMS-387032 has been found to block IL-1β-induced expression as well as steady-state mRNA levels of cyclooxygenase 2, a protein providing a survival advantage to transformed cells through the inhibition of apoptosis, increased attachment to the extracellular matrix, increased invasiveness, and stimulation of angiogenesis, indicating a novel target for BMS-387032 (Mukhopadhyay et al. 2006).

23.5.2.5 SCH 727965 (Dinaciclib)

SCH 727965 is an extremely potent and selective CDK inhibitor, which in vitro inhibits the activity of multiple CDKs at low nanomolar levels (cyclin A–CDK2, $IC_{50} = 1$ nM; p35–CDK5, $IC_{50} = 1$ nM; cyclin T–CDK9, $IC_{50} = 1$ nM; cyclin B–CDK1, $IC_{50} = 4$ nM) (Parry et al. 2010). SCH 727965 exhibits superior activity with an improved therapeutic index. In cell-based assays, SCH 727965 completely suppressed pRb phosphorylation, which correlated with apoptosis onset and total inhibition of 5-bromo-2′-deoxyuridine incorporation in 106 tumor cell lines (including 60 lines from NCI) with diverse origin and genetic background. SCH 727965 induces regression of established solid tumors in a range of mouse models, associated with modulation of pharmacodynamic biomarkers (e.g., S807/S811 phosphorylated pRb) in skin punch biopsies and rapidly reversible, mechanism-based effects on hematologic parameters. Apoptosis occurs prior to cell cycle arrest after exposure to SCH 727965, suggesting that inhibition of transcriptional CDK9 plays an important role in killing tumor cells. SCH 727965 is in multiple phase 1 and 2 trials in patients with advanced cancers, including solid tumors (e.g., breast and non-small-cell lung cancers) and hematopoietic malignancies (e.g., non Hodgkin's lymphoma, multiple myeloma, chronic lymphocytic leukemia), administered as an intravenous infusion on days 1, 8, and 15 of each 28-day cycle (Johnson et al. 2012; Feldmann et al. 2011; Bates et al. 2011; Fu et al. 2011).

23.5.3 Molecular Mechanism of Action Driven Combinational Targeted Therapy

In view of the molecular mechanism of action (MMOA) of agents that target CDK-regulating events such as the cell cycle and transcription, efforts to combine CDK inhibitors with other targeted agents in various types of human cancer have been the focus of attention. Some representative cases are summarized as in the following sections.

23.5.3.1 Flavopiridol

Synergistic interactions between flavopiridol and TRAIL have been described by several groups. For example, it is reported that synergism between flavopiridol and TRAIL in human leukemia cells stems from downregulation of XIAP (Rosato et al. 2004). Such studies provide a rationale for future attempts to combine CDK inhibitors with TRAIL in leukemia and lymphoma therapy.

The preclinical studies demonstrate that flavopiridol synergistically enhances anticancer activity of pan-Bcl-2 antagonists (or BH3 mimetics) such as HA14-1 (Pei et al. 2004) and obatoclax (Chen et al. 2012). The MMOA includes reactive oxygen species generation dependent activation of the stress-related JNK pathway (Pei et al. 2004), and imbalance between antiapoptotic (e.g., downregulation of Mcl-1 and Bcl-xL) and proapoptotic (e.g., upregulation of the BH3-only protein Bim, NBK/Bik, Noxa) machinery (Chen et al. 2012).

There is also evidence that flavopiridol interacts synergistically with histone deacetylase inhibitors (HDACIs) to induce apoptosis in human leukemia cells. This concept was driven by the findings that interference with $p21^{Cip}$ expression and resulting cell differentiation (e.g., induced by phorbol 12-myristate 13-acetate) by flavopiridol results in a marked increase in apoptosis (Cartee et al. 2002). For example, a highly synergistic interaction between flavopiridol and the HDACI vorinostat (suberoylanilide hydroxamic acid) was observed in human leukemia cell lines as well as primary AML blasts (Almenara et al. 2002). This interaction stemmed in part from flavopiridol-mediated inhibition of $p21^{CIP1}$ induction, an event known to promote the lethality of HDACIs. On the basis of these findings, a phase I trial of vorinostat, administered at 200 mg per os three times daily for 14 days in conjunction with flavopiridol administered as a 1-h infusion daily (days 1–5) in patients with refractory AML/high-risk myelodysplastic syndrome has been initiated and is ongoing, along with plans to incorporate the new infusional flavopiridol schedule. Moreover, mechanistic studies on interactions between flavopiridol and HDACIs have also led to new insight into a critical role of the NF-κB signaling pathway in tumor cell response to HDACIs (Dai et al. 2003b; Gao et al. 2004). Further, it has been found that disruption of HDACI-induced NF-κB activation strikingly increases the lethality of HDACIs (Dai et al. 2005a, 2011b).

Based on these findings, multiple clinical trials have been initiated to test this novel therapeutic strategy (Dai et al. 2008a, 2011c).

On the basis of evidence of synergism between flavopiridol and the Bcr/Abl kinase inhibitor imatinib mesylate in chronic myeloid leukemia cells, including some resistant to imatinib (Kasten and Giordano 2001), a phase I trial of imatinib mesylate and flavopiridol has been initiated. Although the maximum tolerated dose for this combination was not identified, further efforts in this direction have been deferred in light of the introduction of second-generation Bcr/Abl kinase inhibitors (e.g., dasatinib, nilotinib).

Finally, on the basis of evidence of synergistic interactions between flavopiridol and the proteasome inhibitor bortezomib in malignant hematopoietic cells (Dai et al. 2003c, 2004b), a phase I trial has been initiated in patients with refractory multiple myeloma and indolent no-Hodgkin's lymphoma in which escalating doses of flavopiridol and bortezomib are given as an intravenous infusion on days 1, 4, 8, and 11 of a 21-day cycle. The regimen has proven to be well tolerated, and the maximum tolerated dose has not yet been reached. Notably, several patients who either longer responded to bortezomib have responded to the combination of bortezomib and flavopiridol (Holkova et al. 2011). Two complete responses (12 %) and five partial responses (31 %) were observed at the maximum tolerated dose (overall response rate of 44 %) (Holkova et al. 2011).

23.5.3.2 CYC202

CYC202 interacted synergistically with bortezomib in human multiple myeloma cells (Raje et al. 2005), but whether the mechanism underlying this interaction is the same as that responsible for flavopiridol/bortezomib synergism in human leukemia cells, and whether such findings can be extended to leukemia and lymphoma, remains to be determined.

Synergy between roscovitine and the pan-HDACI LAQ824 has been described in human leukemia cells, and this interaction was related to downregulation of Mcl-1, $p21^{CIP1}$, and XIAP, as well as induction of oxidative injury (Rosato et al. 2005).

Roscovitine displays highly synergistic interactions with the Bcl-2 antagonist ABT-737 in human leukemia cells (Chen et al. 2007b). The mechanism responsible for this interaction was determined to be roscovitine-mediated Mcl-1 downregulation, which cooperated with disruption of the function of Bcl-xL by ABT-737 to unleash Bak and activate Bax. In view of evidence of in vivo activity of agents such as ABT-737 in murine models of lymphoma (Oltersdorf et al. 2005), the concept of combining Bcl-2 antagonists with CDK inhibitors warrants further attention.

It is also noted that the PI3K/Akt pathway plays a functional role in regulating the apoptotic response of human leukemia cells to pharmacological CDK inhibitors (including roscovitine and flavopiridol), whereas combined interruption of

CDK- and PI3K-related pathways significantly increases therapeutic activity in hematological malignancies (Yu et al. 2003).

23.5.3.3 UCN-01

In addition to combination strategies involving UCN-01 and conventional chemotherapy (Harvey et al. 2001), investigations of UCN-01 have focused on interactions with inhibitors of the Ras/Raf/MEK1/2/ERK1/2 pathway. Exposure of human leukemia or multiple myeloma cells to UCN-01 results in activation of MEK1/2/ERK1/2, and interference with the latter process, i.e., by MEK1/2 inhibitors such as PD184352, results in a dramatic increase in apoptosis (Dai et al. 2001, 2002b; Yu et al. 2002a). Similar synergistic interactions between MEK1/2 inhibitors and UCN-01 were observed in human solid tumors, including breast cancer, prostate carcinoma (McKinstry et al. 2002; Hawkins et al. 2005; Hamed et al. 2008), and glioblastoma (Tang et al. 2012a). These events were associated with enhanced activation of CDK1 (Pei et al. 2006), consistent with the ability of UCN-01 to inhibit Chk1. However, this regimen is also able to kill cytokinetically quiescent (G0/G1) human malignant cells (Pei et al. 2011). Such a finding argues against a notion that the activity of this combinational therapy is only restricted to cycling cells. It also raises the possibility that this regimen may be active against cytokinetically quiescent cancer stem cells.

Lethality of the UCN-01/MEK1/2 inhibitor regimen primarily involved activation of the intrinsic, mitochondrial pathway likely via upregulation of the BH3-only protein Bim (Pei et al. 2007), and was substantially blocked in cells overexpressing Bcl-2 or Bcl-xL. However, lethality of the regimen was restored in leukemia cells by agents capable of activating the extrinsic, apoptotic pathway, e.g., TRAIL (Dai et al. 2003a). Similar events were observed in human hematopoietic malignant cells exposed to UCN-01 in conjunction with agents targeting Ras, such as farnesyltransferase inhibitor (Dai et al. 2005b, 2008b; Pei et al. 2005), statins (Dai et al. 2007), or Src (Dai et al. 2011a; Mitchell et al. 2011). UCN-01 activity was also shown to be dramatically enhanced by the mammalian target of rapamycin inhibitor rapamycin (Hahn et al. 2005), NF-κB inhibitors (Dai et al. 2004a), or poly(ADP-ribose) polymerase inhibitors (Tang et al. 2012b). Combining UCN-01 with multiple agents (particularly those targeting the Src/Ras/Raf/MEK/ERK pathway) is a good example for development of an MMOA-driven rationale, that is, to combine the cell cycle modulators with other targeted agents that block the activation of the compensatory survival pathway in tumor cells exposed to the former, an important mechanism responsible for acquired drug resistance toward most anticancer agents, including the novel small-molecule inhibitors (Dai and Grant 2010a, b; Dent et al. 2011). This concept is further supported by later observations that combining inhibitors of Aurora kinases that regulate cell cycle transit from G2 phase through cytokinesis with other

targeted agents (e.g., HDACIs) markedly increases the anticancer activity (Dai et al. 2008c; Nguyen et al. 2011).

23.6 Future Perspectives

Targeting cell-cycle-regulatory, transcriptional, neural CDKs is a highly attractive approach in cancer treatment as well as neuron protection, in which dysregulation of CDKs or their regulatory partners (e.g., cyclins, cyclin-like proteins, and endogenous CDK inhibitors) appears to play an important role in disease pathogenesis and prognosis (Dai and Grant 2004). The recent advances in methods allows us to gain deeper insight into the MMOA of pharmacological CDK inhibitors as well as other cell cycle modulators (Dai and Grant 2011). These MMOA are way beyond the original consideration of CDK inhibition (Dai et al. 2002a), but are related to multiple novel mechanistic aspects, including apoptosis induction, transcription inhibition, downregulation of various short-lived proteins (e.g., cyclin D1, Mcl-1, and VEGF), and antiangiogenesis. (Dai and Grant 2006). Better understanding of MMOA has been providing fundamental power to drive recent rapid development of new-generation, more potent and more selective small-molecule CDK inhibitors. Several of them, such as CYC202 (seliciclib), BMS-387032 (SNS-032), SCH 727965 (dinaciclib), PD-0332991, P-276-00, R-547, AZD5438, ZK 204709, PHA-848125, and AT7519 have entered clinical trials, and many others are in preclinical development with considerable interest.

Single-agent activity of CDK inhibitors in cancer has been limited to date, although many of them appear to have significant activity in preclinical settings. Significantly, the MMOA-driven rationale of combining CDK inhibitors with conventional chemotherapeutic drugs and, importantly, other novel targeted agents offers the potential for enhanced tumor-selective cytotoxicity and circumvention of drug resistance. In fact, the ultimate role of CDK inhibitors as anticancer therapeutics may be as modulators or sensitizers for conventional chemotherapy and/or novel agents. In a context related to this, one overarching question is whether to target single or multiple CDKs for cancer therapy. Despite great efforts, the development of monospecific CDK inhibitors has not succeed so far in cancer treatment. Neoplastic cells are characterized by uncontrolled proliferation due to constitutive activation of cell-cycle-regulatory CDKs (e.g., CDK4/CDK6, CDK2, and probably CDK1 as well) owing to abnormalities of their moderators (e.g., cyclins and endogenous CDK inhibitors). Moreover, rapid growth of transformed cells requires continuous transcriptional activity of RNA pol II to ensure de novo protein synthesis via gene expression. Therefore, therapeutic intervention that interrupts cell cycle progression and global transcription by inhibition of both cell-cycle-regulatory and transcriptional CDKs is a very sound rationale in the treatment of cancer (Wesierska-Gadek and Kramer 2012). Therefore, the highly heterogeneous characteristics of human cancer may not only necessitate the pleiotropic effects of pan-CDK inhibitors, but may also warrant the simultaneous

interference with multiple pathways by combining CDK inhibitors with conventional chemotherapy as well as other targeted agents.

Most, if not all, currently available CDK inhibitors hit at least CDKs. Theoretically, targeting a single CDK may improve the selectivity of therapy, thus minimizing toxicity by preventing healthy cells from experiencing undesired side effects. In the case of neuron protection, although "inappropriate" activation of cell-cycle-regulatory CDKs generally leads to neuronal death (Rashidian et al. 2005), global inhibition of multiple CDKs by pan-CDK inhibitors may be not beneficial, and is probably harmful, because they also inhibit CDK5, the only postmitotic neural CDK, which is critical for various aspects of nervous system development and functions (e.g., neuronal migration, neuronal survival, dendritic spine formation, synaptogenesis, adult neurogenesis, neurotransmission, homeostatic plasticity, and learning and memory) (Ou et al. 2010; Park et al. 2011). Thus, it is plausible that the monospecific CDK inhibitors might be more beneficial in the treatment of disorders such as cardiac hypertrophy and neurodegenerative diseases, in which a single CDK is dysregulated. In this context, therapeutic inhibition of the hyperactivated CDK9 or CDK5 may be sufficient to reduce the enhanced transcription required for hypertrophic cardiomyocytes or to prevent extensive neuron apoptosis in neurodegenerative diseases, respectively.

In summary, pan-CDK inhibitors might be more effective in the treatment of cancer, as multiple, rather than single, pathways are deregulated in malignancies. In contrast, inhibition of a single CDK (e.g., CDK4 or CDK5) may provide more benefit for neuron protection in stroke or neurodegeneration, respectively. However, since the normal and abnormal roles of CDK5 are dependent on its non-cyclin partners, p35/p39 versus p25, it will be ideal to develop small-molecule inhibitors that specifically target p25–CDK5, rather than p35/p39–CDK5, for treatment of neurodegenerative diseases. Therefore, therapeutic development and application of CDK inhibitors, like that of most targeted agents, should be tailored to the individual patient by use of genetic and other information, a model termed "personalized medicine" (Roychowdhury et al. 2011).

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