FUTURE ASPECTS OF TUMOR SUPPRESSOR GENE

Edited by **Yue Cheng**

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Preface

Losses of specific chromosomal regions are frequently reported in different human tumors, suggesting that these regions may contain important genes associated with tumor development. Cell fusion studies provided the first functional evidence for a class of negatively-acting tumor suppressor genes (TSGs) harbored on certain human chromosomes. Based on Knudson's "two-hit hypothesis", the first TSG, RB was identified. Since 1980s, many TSGs have been discovered by using different approaches. Accumulated knowledge indicates that TSGs not limited to tumor suppression play critical roles in various biological activities in human cells.

In 20132, InTech published a book called "Tumor Suppressor Genes", which covers the most important fields, from cell cycle control, signaling pathways, epigenetic regulation, and current challenges to therapeutic applications of known TSGs. Some well-studied TSGs, such as p53 and p16, and their regulatory mechanisms in tumor development are addressed in this book. However, TSG research is a fast growing area, and many novel approaches and findings have been discovered recently. Therefore, it is necessary to publish a new open access book that may provide future directions for TSG studies.

This book, "Future Aspects of Tumor Suppressor Genes", contains some important areas that were not mentioned in the previous book. The majority of known TSGs were identified from hereditary tumor syndromes. However, more than 90% of human tumors are sporadic cases, so it is always a challenge to identify tumor susceptibility loci in sporadic tumors. Using animal models, authors in this book investigated whether strain-specific allele loss was an important clue to identify tumor suppressors involved in tumor susceptibility, which should be interesting to many researchers. Other basic researches contain investigations of several TSG signaling pathways from different laboratories: START-GAP/DLC family proteins and their molecular pathways involved in the control of cell growth, E2F-mediated tumor suppressive mechanism associated with RB, p53, ARF, p27Kip1 and TAp73 transcription factors, and TSGs in the regulation of receptor tyrosine kinase signaling via a negative feedback loop. Understanding these signaling regulatory mechanisms may lead to findings of molecular targets for cancer therapy. In recent years, it has been well-accepted that microRNAs are an abundant class of endogenous small RNA molecules that can regulate tumor development. To reflect the trends of these novel researches, authors in this book present an extensive review for current knowledge of microRNAs that play in the control of tumor growth and therapeutic application.

This book also includes some other fascinating fields and emerging subjects in TSG studies. For example, the application of Drosophila as a special model for tumor suppression studies is addressed, and future directions used for the pharmacological screening and therapy

strategies are also proposed. Natural compounds, such as polyphenols, interfere with the initiation and progression of cancer development via multiple TSG pathways. Recent evidence, demonstrating that these compounds are able to modulate various cellular activities, such as cell cycle arrest, anti-angiogenesis, and metastasis suppression, are summarized in the relevant chapter. Finally, the regulatory role of TSGs, such as p16, p53 and RB, in cell reprogramming, stemness transition process, and signaling networks of these genes during these cellular processes are extensively reviewed, which indicates that TSGs are actively involved in many aspects of stem cell biology and regenerative medicine.

I would like take this opportunity to express my gratitude to all authors and InTech staff for their contributions in this publication project, and I hope that this book will be helpful for students, researchers and clinicians.

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Strain-Specific Allele Loss: An Important Clue to Tumor Suppressors Involved in Tumor Susceptibility

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Additional information is available at the end of the chapter

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1. Introduction

Development of tumors is controlled by multiple genes such as cellular oncogenes and tumor suppressors activated or inactivated by somatic mutations and/or epigenetic mechanisms. Tumor development is also controlled by heritable factors as well as environmental factors, i. e., diet, oxidative stress and sustained inflammation, as reviewed by a large number of recent reports [1-12]. Both heritable and environmental factors are important targets for clinical controls and prevention of cancers.

Heritable factors underlying cancer risks have been identified in familial cancer-prone pedigrees. In the pedigree members, tumors develop in a Mendelian dominant inheritance fashion. Breast cancer 1, early onset (*BRCA1*) encoding a nuclear phosphoprotein that plays a role in maintaining genomic stability is one of the heritable cancer risk factors hitherto identified. Women bearing a mutated *BRCA1* allele are at high risk for both breast and ovarian cancers through their lifespan. According to the recent estimations, average cumulative risks in *BRCA1*-mutation carriers by age 70 years are 65% (95% confidence interval 44%–78%) for breast cancer and 39% (18%–54%) for ovarian cancer [13]. Thus, disease penetrance is incomplete, albeit rather high, in the mutated-*BRCA1* carriers. The *BRCA1* gene maps to human chromosome 17q21, where frequent loss of heterozygosity (LOH) is observed in both familial and sporadic breast cancers. Although tumors developed in the *BRCA1*-mutation carriers are homozygous for the defective *BRCA1* allele via LOH mechanisms, sporadic cases rarely show mutation in the *BRCA1* gene [14]. The *BRCA1* gene may rather undergo inactivation via epigenetic mechanisms such as DNA methylation in sporadic tumors.

Unlike the *BRCA1* case, tumor susceptibility is expressed in a non-Mendelian inheritance manner, because multiple genes with incomplete penetrance participate in the phenotype. Moreover, tumor susceptibility alleles may occasionally express genetic interaction, i. e.,

epistasis that hides or enhances the effect of some alleles at some susceptibility loci with the effect of other alleles at other susceptibility loci [15]. Despite growing number of association studies localizing tumor susceptibility loci exploiting SNPs in humans [16, 17], validation of these loci in human population with miscellaneous variations in the genetic background might be an intractable task.

Several strains of mice with different susceptibility to lymphomagenesis so far reported might be useful in the study of tumor susceptibility. Using genetic crosses between BALB/cHeA (refer to as BALB/c, hereafter) and STS/A (refer to as STS) mice with different tumor susceptibility, and between the BALB/c and recombinant congenic CcS/Dem strains of mice with 12.5% STS and 87.5% BALB/c allele in the genome, we mapped three loci controlling susceptibility to radiation-induced apoptosis of thymocytes to chromosomes 16, 9 and 3 [18, 19], and two loci for susceptibility to lymphomagenesis to chromosome 4 [20]. We identified the protein kinase, DNA activated, catalytic polypeptide (*Prkdc*) as a candidate for the apoptosis susceptibility gene mapped to chromosome 16, which was also associated with susceptibility to radiation lymphomagenesis [21]. As indicated by our studies, susceptibility to apoptosis as well as lymphomagenesis is controlled by multiple genes. To analyze the effect of one gene involved in such multigenic traits, congenic animals are ordinarily used. We are currently analyzing the genes controlling susceptibility to lymphomagenesis on chromosome 4 by the use of congenic animals.

In this chapter, we initially review recent advances in the research of tumor susceptibility, in particular, susceptibility to radiation lymphomagenesis in mice, and show that two loci controlling radiation lymphomagenesis map to chromosome 4. Then, we show that two types of allele loss, i. e., loss common to lymphoma and parental strain-specific loss, occur in radiation-induced lymphomas from various F_1 hybrids between strains with different lymphoma susceptibility. We show that LOH on chromosome 4 in F_1 hybrids between BALB/c and STS occurs in a strain-specific manner and exhibits a bias towards the STS allele loss. At the close, by exploiting congenic strains of mice containing different segments of chromosome 4 from the donor strain STS on the BALB/c background, we present a concordance between the allele loss region and a lymphoma susceptibility locus area on chromosome 4, where the BALB/c mouse harbors a hypomorphic allele of *Cdkn2a*. Significance of the strain-specific allele loss in probing tumor susceptibility loci will be discussed.

2. Mouse strain difference in susceptibility to radiation-induced lymphomagenesis

In laboratory strains of mice irradiated by ionizing radiation according to a well-established protocol, development of lymphomas starts around three months after the exposure to radiation and is terminated around ten months. Radiation-induced lymphomas are mostly of thymic origin. Several laboratory strains of mice such as BALB/c and C57BL reside in *Mus musculus musculus*, and are known to be highly susceptible to radiation-induced lymphomagenesis, while other strains STS and MSM/Ms (refer to as MSM) are not [22, 23]. The BALB/cHeA and STS/A strains of mice are originally provided by Dr. J. Hilgers at the Netherlands

Cancer Institute [22], and maintained more than twenty generations at the animal facility of Osaka Prefecture University. The BALB/cHeOpu mouse is the direct descendant of the BALB/cHeA mouse [20]. The MSM/Ms strain of mice belongs to a subspecies *Mus musculus molossinus*. Its progenitor was trapped in Mishima-city, Shizuoka, Japan and established as an inbred strain at the National Institute of Genetics (Mishima, Japan). Mice were exposed to 4 x 1.7 Gy of X rays using a well-established protocol for radiation-induced lymphomagenesis. Tumor development was observed during one year. The results were summarized in Table 1. BALB/cHeA mice developed lymphomas at high frequency (33/43, 77%), while STS/A mice develop tumors at less than 10% of frequencies [22]. The onset of tumor development was around three months in both strains. On the other hand, one of 30 MSM/Ms mice developed lymphoma with more than ten months of latency. Lymphomas occurred at high frequency (30/35, 86%) in BALB/cHeOpu mice subjected to X-ray irradiation using the same protocol. Thus, the pattern of tumor development in BALB/cHeOpu mice showed good concordance with that in their progenitor BALB/cHeA mice [20].

Strain of mice	Number of irradiated[a]	Number of affected (%)[b]	Reference
BALB/cHeA	43	33 (77%)	[22]
BALB/cHeOpu	35	30 (86%)	[20]
STS/A	60	5 (8%)	[22]
MSM/Ms	30	1 (3%)	[23]

[a]Only females.

[b]Cumulative incidence of lymphomas within one year in BALB/cHeA, STS/A and MSM/Ms, and within ten months in BALB/cHeOpu.

Table 1. Strain difference in susceptibility to radiation lymphomagenesis

3. Current status of the studies on tumor susceptibility in mice

Numerous tumor susceptibility loci have been mapped by analyzing genetic crosses between strains of mice exhibiting different tumor susceptibility [17]. Several genes responsible for tumor susceptibility have been identified, some of which are validated by supporting evidences: *Pla2g2a* encoding phospholipase A2, group IIA (platelets, synovial fluid), for the modifier of *Min1* (*APC^Min*)-induced intestinal tumors (*Mom1*) identified in the distal portion of chromosome 4 [24–26]; cyclin-dependent kinase inhibitor 2A (*Cdkn2a*) encoding a tumor suppressor p16, for pristen-induced plsmacytoma resistance1 (*Pctr1*) mapped in the middle of chromosome 4 [27, 28]; protein tyrosine phosphatase, receptor type, J, (*Ptprj*), for susceptibility to colon cancer 1 (*Scc1*) on chromosome 2 [29, 30]. LOH occurs at *PTPRJ*, the human homolog of mouse *Ptprj* (*Scc1*), in the early stage of human colorectal cancer [31]. Hence, *PTPRJ* may play a role in tumor suppression in humans. The biological function of *Pla2g2a* (*Mom1*) differs from other tumor susceptibility genes so far identified. *Pla2g2a* plays a role in physiological processes such as anti-bacterial defense, inflammation and eicosanoid generation, which are preferable targets of medical controls for cancer prevention.

Despite the availability of strains of mice with obvious difference in susceptibility to radiation lymphomagenesis, it is much difficult to analyze such traits as to be expressed in a binominal fashion (tumor-free survivals of animals after exposure to radiation). However, there is one successful case: a suggestive linkage near D4Mit12 at 57.8 centimorgan (cM) position on chromosome 4 with susceptibility to radiation lymphomagenesis, which was detected in the genetic cross between BALB/c and MSM, is confirmed by exploiting congenic mice with the MSM allele at D4Mit12 on the BALB/c background [32, 33]. Because BALB/c mice had a hypomorphic allele at the *Mtf1* locus, they reported the metal-responsive transcription factor-1 (*Mtf1)* gene as the candidate gene for the susceptibility locus near D4Mit12 [32, 34]. *Mtf1* activates expression of metallothionein I and II genes as well as gamma-glutamylcysteine synthetase, a key enzyme for glutathione biosynthesis, and metallothionein and glutathione are involved in detoxification processes, such as scavenging reactive oxygen intermediates generated by ionizing radiation. Reduced reactivity of Mtf-1 retains an increased level of ROS in the BALB/c thymus [35].

4. Mapping of lymphoma susceptibility loci on mouse chromosome 4 using genetic crosses between BALB/c and STS strains of mice

We so far showed that the protein kinase, DNA activated, catalytic polypeptide (*Prkdc*) gene was a candidate for the apoptosis susceptibility gene on chromosome 16, and also responsible for susceptibility to radiation lymphomagenesis [21]. DNA-PK is a key enzyme for DNA double-stranded-break repair as well as V(D)J recombination of T- and B-cell receptors. Because BALB/c mice carry a *Prkdc* variant allele that causes lower DNA-PK activity, resultant hypersensitivity to radiation may raise frequency of cell death in the thymus and promote lymphomagenesis possibly via illegitimate recombination mechanisms. However, strain difference between BALB/c and STS in susceptibility to radiation lymphomagenesis has not been fully explained by the variations in *Prkdc*. According to M. Okumoto *et al.* [22, 23], cumulative incidence of lymphomas in (BALB/c x STS)F_1 exposed to 4 x 1.7 Gy of X-ray irradiation was in between those in parental BALB/c and STS, while (BALB/c x MSM)F_1 developed lymphomas at high frequency similar to BALB/c. The data suggest that strain difference in tumor susceptibility is controlled by multiple genes that influence onset, latency and frequency of tumorigenesis.

Previously, M. Okumoto *et al.* reported a suggestive linkage of susceptibility to radiation-induced lymphomagenesis, named lymphoma resistance *(Lyr)* (Mouse Genome Informatics, MGI: 96893) in the middle area of chromosome 4 using a series of recombinant inbred (RI) CXS strains of mice whose genome was constituted of 50% STS and 50% BALB/c genes [36]. It is worthwhile to test whether the *Lyr* locus is segregated in a genetic cross using BALB/c and STS. We performed genome-wide screen for microsatellite markers linked to lymphoma susceptibility using siblings from (BALB/c x STS)F_1 backcrossed to BALB/c or STS. We detected significant linkage disequilibrium in the middle area of chromosome 4 by the use of 219 siblings from (BALB/c x STS)F_1 backcrossed to BLB/c [20]. No significant linkage was detected by using another backcross. The primary locus with a conspicuous effect existed in an approximately 10 cM segment spanning D4Mit302 (37.6 cM) and D4Mit255 (48.5 cM) in the middle range of

chromosome 4 (χ^2=19.3, genome-widely corrected p=0.0075). This locus was likely identical to the *Lyr* locus localized between tyrosinase-related protein 1 (*Tyrp1*) (38 cM) and interferon alpha (*Ifna*) (42.6 cM) [36]. The secondary locus with a weaker effect was detected near D4Mit17 (χ^2=16.0, genome-widely corrected p=0.034), a marker approximately 10 cM proximal to D4Mit302. The STS allele at these loci was associated with resistance to lymphomagenesis. *Mtf1*, a candidate susceptibility gene for radiation lymphomagenesis so far identified by other investigators, is located near D4Mit12 (57.8 cM), more than 10 cM distal to the critical regions containing these loci [32, 33]. Effect of *Prkdc*, which we identified as a lymphoma susceptibility gene by exploiting congenic mice [21], was not detected in tumor-free survivals in these crosses.
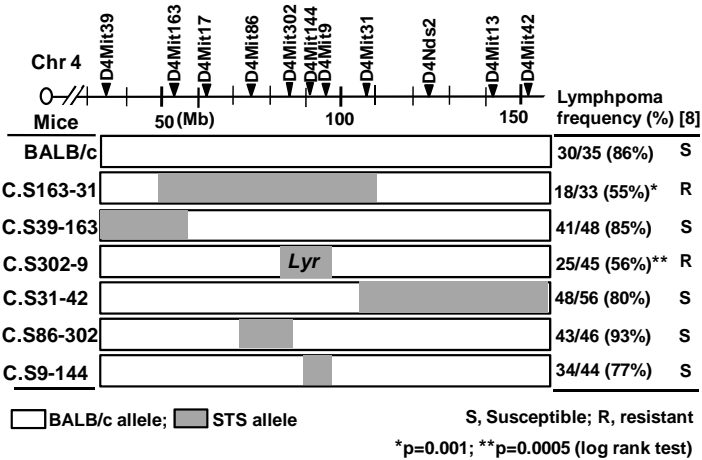
Figure 1. The *Lyr* locus exists between D4Mit302 and D4Mit144 on chromosome 4.

To narrow down the tumor susceptibility gene regions, we generated congenic strains of mice with different portions of STS-derived chromosome 4 on the BALB/c background by back-crossing (BALB/c x STS)F_1 mice to the BALB/c. Establishment of the congenic lines was facilitated by positive and negative selections with typing of microsatellite markers on chromosome 4 and markers distributed in the whole genome [20]. Because the *Lyr* locus was so vicinal (10 cM distance, approximately) to the secondary locus, we selected several strains with or without the STS allele at the critical markers D4Mit17, D4Mit302 and other markers near these markers, and compared their tumor-free survivals with that of BALB/cHeOpu exposed to X-ray irradiation (data shown in Table 1). A part of the results in [20] is represented in Figure 1. In this figure, the strain names of the C.S congenic mice are abbreviated by hyphened two Arabic numbers that represent STS allele-bearing microsatellite (Mit) markers at the proximal and distal end of the chromosomal segment. For instance, C.S163–31 represents a congenic line with the STS allele in the segment spanning D4Mit163 and D4Mit31. The order and megabase (Mb) positions of the markers are indicated by arrowheads on chromosome 4, which is represented by a line at the top of the figure. The primary lymphoma susceptibility

locus *Lyr* exists between D4Mit302 (85.2 Mb) and D4Mit9 (94.7 Mb). Although the secondary locus was not detectable by a simple comparison of the tumor-free survival of congenic lines with that of BALB/c, linkage was reconfirmed by crossing congenic lines (data not shown here).

5. Loss of heterozygosity (LOH) in radiation-induced lymphomas from various F_1 hybrids: common loss and cross-dependant loss

Tumor suppressors frequently undergo loss of heterozygosity (LOH) in a variety of tumors in humans and mice. We previously reported that frequent LOH (more than 20%) occurred on chromosomes 4, 12 and 19 in radiation-induced lymphomas from (BALB/c x STS) F_1 mice, with incidences 27% (20 of 74 lymphomas), 57% (42 of 74 lymphomas) and 50% (37 of 74 lymphomas) on chromosomes 4 (at D4Mit31), 12 (at D12Mit17) and 19 (at D19Mit11), respectively [37] (Table 2). Importantly, STS allele-specific loss occurred on chromosome 4. The bias was confirmed using reciprocal F_1 hybrids between BALB/c and STS [37].

Mice[a]	Number of tumors	Chr	Marker (Mb)[b]	LOH (%)	Reference
(CXS)F_1	74	4	D4Mit31 (106.8)	20 (27%)	[37]
		12	D12Mit17[c]	42 (57%)	
		19	D19Mit11 (42.5)	37 (50%)	
(SXM)F_1	20	4	D4Mit54 (137.4)	5 (25%)	[39]
		12	D12Mit233 (109.5)	12 (60%)	
(CXM)F_1 [d]	81	12	D12Mit181 (110.0)	53 (65%)	[40]
		16	D16Mit122 (74.5)	38 (45%)	

[a]Abbreviations used are BALB/c, C; MSM, M; STS, S.

[b]Megabase (Mb) positions of markers are according to Mouse Genome Informatics (MGI) 5.10.03. (http://www.informatics.jax.org/).

[c]Physical position not assigned.

[d]F_1 hybrids between BALB/c and MSM hemizygous for *Trp53*.

Table 2. LOH in radiation-induced lymphomas from various F_1 hybrids.

In these crosses, allele loss involved almost entire chromosomes 4 and 19, without showing any peaks in LOH frequencies. Cytogenetic analysis showed that allele loss in such large areas was not caused by chromosomal deletion, but ascribable to mitotic recombination [38]. In lymphomas from (STS x MSM)F_1 mice, LOH occurred on chromosomes 4 and 12 with incidences 25% (5 of 20 lymphomas) and 60% (12 of 20 lymphomas) on chromosome 4 (at D4Mit54) and chromosome 12 (at D12Mit233), respectively [39]. In these lymphomas, LOH on chromosome 19 was infrequent (1/20, 5% at D19Mit63). In radiation-induced lymphomas from (BALB/c x MSM)F_1 mice, allele loss frequently occurred on chromosomes 12 (53/81, 65% at D12Mit181) and 16 (38/81, 45% at D16Mit122) [40].

Interestingly, LOH on chromosome 12 commonly occurred in radiation-induced lymphomas from these three F_1 hybrids, while LOH frequencies on chromosomes 4 and 19 markedly varied. Frequent LOH was detected on chromosome 4 in lymphomas from (STS x MSM)F_1 mice, but not (0/20 at D4Mit13) in lymphomas from (BALB/c x MSM)F_1 mice [40]. LOH on chromosome 19 was infrequent (0/20 and 1/20, at D19Mit63 and D19Mit123) in lymphomas from (STS x MSM)F_1 mice. In the context of LOH on chromosome 19, results were similar in lymphomas from the (BALB/c x MSM)F_1 hybrid. Thus, LOH on chromosomes 4 and 19 occurred in a cross-dependent manner. This suggests that LOH frequencies on these chromosomes are controlled by genetic interaction, possibly between putative tumor suppressors, the locations of which are indicated by LOH, and by genetic variations in the background. Moreover, the situation of LOH on chromosome 4 is somewhat different from that on chromosome 19. We present allele loss frequencies at several markers on chromosome 4 in these lymphomas in Table 3.

Mice[a]	Number of tumors	Marker (Mb)[b]	LOH (%)	References
(CXS)F_1	47	D4Mit17 (63.0)	14 (30%)	[37]
		D4Mit9 (94.7)	14 (30%)	
		D4Mit13 (142.0)	14 (30%)	
(SXM)F_1	20	D4Mit9 (94.7)	1 (5%)	[39]
		D4Mit54 (137.4)	5 (25%)	
(CXM)F_1 [c]	20	D4Nds2 (124.4)	0 (0%)	[40]
		D4Mit13 (142.0)	0 (0%)	
(CXM)F_1	43	D4Mit9 (94.7)	3 (7%)	Unpublished data
	51	D4Mit13 (142.0)	4 (8%)	[41]

[a]Abbreviations used are BALB/c, C; MSM, M; STS, S.

[b]Megabase (Mb) positions of markers are according to Mouse Genome Informatics (MGI) 5.10.03. (http://www.informatics.jax.org/).

[c]F_1 hybrids between BALB/c and MSM hemizygous for *Trp53*.

Table 3. Variation of LOH frequencies at microsatellite markers on chromosome 4 in radiation-induced lymphomas from various F_1 hybrids.

Notably, LOH frequency at D4Mit9 was reduced compared to that at D4Mit54, a marker in the proximity of D4Mit13 and approximately 43 Mb distal to D4Mit9, in lymphomas from (STS x MSM)F_1 hybrid mice. Using lymphomas from (BALB/c x MSM)F_1 mice, we reconfirmed that allele loss at markers D4Mit9 and D4Mit13 on chromosome 4 was very infrequent (3/4 and 4/51 [41], respectively). Because D4Mit9 is located very close to cyclin-dependent kinase inhibitor 2A (*Cdkn2a*) encoding tumor suppressors p16 and p19Arf, *Cdkn2a* is excluded as the putative tumor suppressor for lymphomagenesis in the (STS x MSM)F_1 and (BALB/c x MSM)F_1 backgrounds. Frequent LOH on chromosome 4 and 19 were also reported by other investigators in lymphomas from (C57BL/6JxBALB/cJ)F_1 and (C57BL/6J x RF/J) F_1 hybrid mice [42, 43]. According to the data in [43], strain-specific allele elimination is not found in the LOH on chromosome 4 from (C57BL/6JxBALB/cJ)F_1 mice.

The LOH frequencies at markers on chromosome 12 formed a sharp peak near telomere [41], and a putative tumor suppressor B cell leukemia/lymphoma 11B (*Bcl11b*) was later cloned from the peak [44]. The *BCL11B* tumor suppressor is also involved in human T cell acute lymphoblastic lymphomas [45]. Some of the lymphomas used for the genome-wide screen of LOH were generated in *Trp53* hemizygous (BALB/c x MSM)F_1 mice [40]. Because LOH frequencies at markers on chromosome 16 were markedly varied depending on the status of *Trp53* in (STS x MSM)F_1 mice [39], the high frequency of the LOH on chromosome 16 observed in lymphomas from (BALB/c x MSM) F_1 mice may likewise be explained.

6. The STS allele-specific loss occurred in the Lyr region on chromosome 4

Allele loss on chromosome 4 was significantly biased towards loss of the STS allele in lymphomas from (BALB/c x STS)F_1 mice [37]. It is of interest to examine whether putative tumor susceptibility genes on chromosome 4, which we identified in different regions of chromosome 4, are associated with the strain-specific allele loss on chromosome 4 by using congenic strains of mice with various regions of chromosome 4 from the donor strain STS on the background strain BALB/c, namely the C.S congenic series. LOH was studied in lymphomas generated in (BALB/c x C.S163–31)F_1 and (BALB/c x C.S302–9)F_1 mice. Both C.S163–31 and C.S302–9 strains of mice showed resistance to lymphomagenesis as shown in Figure 1. The C.S163–31 strain harbors the STS allele at two tumor susceptibility loci, one locus near D4Mit17 and the other, *Lyr* in the D4Mit302–D4Mit9 segment. The results are shown in Table 4.

Frequent allele loss at markers in the chromosome 4 segments was detected in lymphomas from (BALB/c x C.S163–31)F_1 (cross A) and (BALB/c x C.S302–9)F_1 (cross B) with incidences 11/34 (32%) and 10/34 (29%), respectively. The LOH frequencies in these F_1 hybrids were concordant with the original data in (BALB/c x STS)F_1 ([37] in Table 3). The STS-allele loss ratios were 9/11 (D4Mit302) and 10/11 (D4Mit9) in the cross A; 8/10 (D4Mit302) and 9/10 (D4Mit9) in the cross B. Because the STS-allele loss occurred with similar ratio in both crosses, we combined the data from crosses A and B (presented as A + B in Table 4). Analysis of the combined ratios 17/21 (D4Mit302) and 19/21 (D4Mit9) indicate that the distortions are significant at both markers D4Mit304 and D4Mit9 ($\chi2$ values were 8.0 and 13.7, p<0.005, degree of freedom = 1, respectively). The data indicating the STS-allele specific loss (D4Mit31) in lymphomas from reciprocal (BALB/c x STS)F_1 and (STS x BALB/c)F_1 hybrids are also presented ([37] in Table 4). Thus, the skewed allele loss that was originally observed in a wide area of chromosome 4 in (BALB/c x STS)F_1 and (STS x BALB/c)F_1 hybrids is reproducible in the limited segments of the STS-derived chromosome 4. Our results suggest that tumor suppressor(s) associated with susceptibility to lymphomagenesis exist in the limited areas of chromosome 4. Since C.S39–86 mice carry the STS allele in the vicinity of D4Mit17, i. e., the secondary locus controlling susceptibility to lymphomagenesis, we further examined allele loss at markers D4Mit7 (67.7 Mb), a marker in the vicinity of D4Mit17, and D4Mit86 using 25 lymphomas from (BALB/c x C.S39–86)F_1 x BALB/c mice [20]. Allele loss at these markers was detected in only one of 25 tumors (less than 5%). In this case the BALB/c allele was lost. Hence, approximately 40 Mb of the D4Mit39–86 segment, to which the secondary locus for tumor susceptibility was

localized, was excluded from the skewed loss region. Analysis on congenic strains strongly suggest that the STS-strain specific loss is ascribable to the D4Mit302–D4Mit9 segment of chromosome 4, which harbors a putative tumor susceptibility gene *Lyr*.

Mice[a]	Number of tumors	Marker (Mb)[b]	LOH (%)	S loss	C loss
A. (C x C.S163–31)F$_1$	34	D4Mit17 (63.0)	11 (32%)	9	2 [c]
		D4Mit302 (85.2)	11 (32%)	9	2 [c]
		D4Mit9 (94.7)	11 (32%)	10	1 [c]
		D4Mit31 (106.8)	11 (32%)	10	1 [c]
B. (C x C.S302–9)F$_1$	34	D4Mit302 (85.2)	10 (29%)	8	2 [d]
		D4Mit9 (94.7)	10 (29%)	9	1 [d]
A + B	68	D4Mit302 (85.2)	21 (31%)	17	4
		D4Mit9 (94.7)	21 (31%)	19	2
(C x S)F$_1$	39	D4Mit31 (106.8)	11 (28%)	10	1 [e]
(S x C)F$_1$	35	D4Mit31 (106.8)	9 (26%)	7	2 [e]
(C x S)F$_1$ + (S x C)F$_1$	74	D4Mit31 (106.8)	20 (27%)	17	3 [e]

[a]BALB/c and STS mice are abbreviated as C and S.

[b]Megabase (Mb) positions of markers are according to Mouse Genome Informatics (MGI) 5.10.03. (http://www.informatics.jax.org/)

[c]Unpublished data.

[d] Data in [20].

[e]Data in [37].

Table 4. LOH at markers on chromosome 4 in lymphomas induced by radiation in F$_1$ hybrids between BALB/c and STS or C.S congenic lines.

In the *Lyr* region between D4Mit302 and D4Mit144, three known tumor suppressors *Cdkn2a*, cyclin-dependent kinase inhibitor 2B (*Cdkn2b*) encoding p15INK4B and methylthioadenosine phosphorylase (*Mtap*) exist (Figure 2). Involvement of *Cdkn2a* and *Cdkn2b,* specifically in acute lymphoblastic lymphomas (ALL) in humans and thymic lymphomas in mice has been reported [46–49]. Mtap is a key enzyme in purine and polyamine metabolism and regulation of transmethylation reactions and frequently inactivated in human tumors such as lymphomas by large homozygous deletion of the 9p21 region [50]. Since these deletions inactivate *CDKN2A/ARF* and *CDKN2B* as well as *MTAP* [51], it has been hypothesized that loss of *MTAP* in tumors is a result of co-deletion. However, a recent study showed that mice heterozygous for the targeted *Mtap* gene were affected with T-lymphocyte hyperproliferation followed by T-cell lymphomas late in their lives [52]. In these lymphomas, as shown by the study, expression of *Mtap* was markedly reduced, while expression of *Cdkn2a* was not. The results suffice the criteria for tumor suppressors, indicating that *Mtap* is a candidate tumor suppressor distinct from *Cdkn2a*. It has also been reported that the *Cdkn2b* gene is particularly inactivated by allele loss and hypermethylation of the remainder allele in radiation-induced lymphomas in mice [53]. BALB/c mice carry a hypomorphic variant allele at *Cdkn2a*, which is shown to be

causative in the sensitivity to plasmacytomagenesis [28]. STS mice and most of strains other than BALB/c have the wild-type allele at the *Cdkn2a* locus ([28], DNA sequences we confirmed). Although *Cdkn2a* is a potential candidate for tumor susceptibility gene *Lyr*, *Cdkn2b* and *Mtap* are at present not ruled out as candidates for the tumor susceptibility gene. Analysis for allele loss, sequences and expression levels of these tumor susceptibility genes in BALB/c and C.S302–9 congenic mice is currently underway.
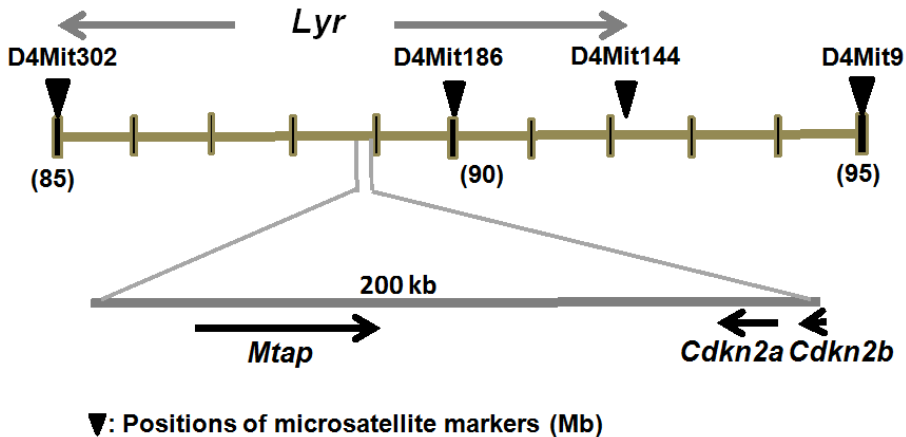
Figure 2. Locations of tumor suppressors in the lymphoma susceptibility *Lyr* gene region.

7. Conclusion

Frequent LOH occurs on chromosomes 4, 12 and 19 in radiation-induced lymphomas from various F_1 hybrid mice. These allele losses are classified into two groups: common loss and cross-dependent loss. The putative tumor suppressor harbored in common loss on chromosome 12 might be a key player in radiation-induced lymphomgenesis. Cross-dependent allele loss such as those on chromosomes 4 and 19 reflects genetic interaction between tumor suppressors harbored in the LOH region and the genetic background. BALB/c and STS strains of mice are susceptible and resistant to radiation-induced lymphomagenesis, respectively. Allele loss occurs on chromosome 4 in approximately 30% of lymphomas induced by radiation in (BALB/c x STS)F_1 mice and shows preferential loss of the STS allele. Our analysis of congenic lines with various portions of STS-derived chromosome 4 on the BALB/c background shows a link between the skewed LOH and the tumor susceptibility *Lyr* locus, where tumor suppressors *p16Ink4a/Arf*, *p15Ink4b* and *Mtap* genes are localized. Although the *Lyr* gene is as yet unidentified, *p16Ink4a/Arf* may be one of the potential candidates. Studying cross-specific LOH and distorted allele loss may lead to better understanding of variable pathways of radiation lymphomagenesis.

Acknowledgements

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To Grow, Stop or Die? – Novel Tumor-Suppressive Mechanism Regulated by the Transcription Factor E2F

Eiko Ozono, Shoji Yamaoka and Kiyoshi Ohtani

Additional information is available at the end of the chapter

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1. Introduction

Proliferation of mammalian cells is strictly regulated by growth stimulation. Cell proliferation is stimulated not only by normal growth stimulation but also by abnormal growth stimulation originated from oncogenic changes. Such abnormal growth stimulation leads to tumorigenesis, if not properly guarded by appropriate cellular response. Cells are endowed with intrinsic tumor suppressor pathways to protect cells from tumorigenesis upon such oncogenic threat [1]. The tumor suppressor pathways halt cell proliferation either by restraining cell cycle progression or by inducing apoptosis (programmed cell death) in case of being unable to stop aberrant cell cycle progression. Consequently, the cell-fate, whether to grow, stop growing or die, is dependent on the balance between growth-promoting effects originated from oncogenic changes and growth-suppressive effects mediated by the tumor suppressor pathways upon oncogenic changes (Figure 1). When the tumor suppressor pathways are disabled by further oncogenic changes, the balance of cell-fate determination shifts from growth suppression to proliferation, and cells start deregulated proliferation, leading to tumorigenesis. Among the intrinsic tumor suppressor pathways, two major pathways are the RB pathway and the p53 pathway. Both pathways are important for induction of cell cycle arrest or apoptosis [2]. In addition, accumulating evidence indicates that the tumor suppressor TAp73, a member of the p53 family, also plays crucial roles in tumor suppression by inducing apoptosis independent of p53 [3, 4].

The transcription factor E2F, the main target of the RB pathway, plays crucial roles in cell cycle progression by activating growth-promoting genes [5]. In this regard, E2F is thought to mediate growth-promoting effects originating from normal growth stimulation and oncogenic changes. Supporting this notion, E2F could be an oncoprotein [6]. On the other hand, recent studies indicate that E2F also plays crucial roles in activation of the major intrinsic tumor suppressor path-

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ways by sensing oncogenic changes, halting cell proliferation by inducing cell cycle arrest or apoptosis. Supporting this notion, E2F could also be a tumor suppressor [7]. Taken together, these observations indicate that E2F is located at the center of the balance between cell proliferation and cell cycle arrest or apoptosis, determining the cell-fate upon oncogenic changes (Figure 1). E2F can be regarded as a double-edged sword in cell growth control. In this chapter, we will describe the major intrinsic tumor suppressor pathways and activation of the tumor suppressor pathways by E2F upon oncogenic changes. We will focus on how E2F differentially regulates expression of target genes upon normal growth stimulation and oncogenic changes that have completely opposite roles in cell-fate determination, to grow, stop or die.
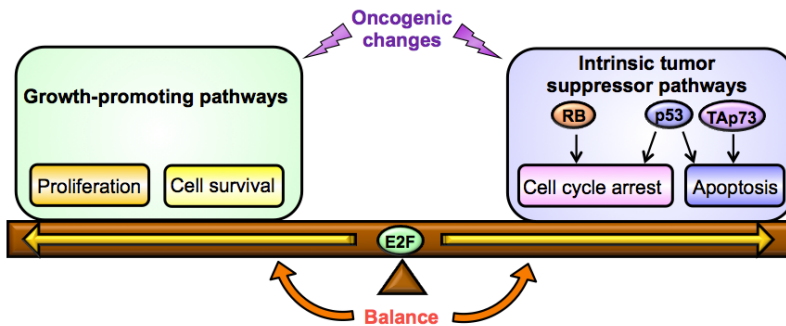
Figure 1. To grow, stop or die? The balance between the activity of growth-promoting pathways and tumor suppressor pathways determines the cell fate upon oncogenic changes. E2F is located at the center of the balance (see also Figure 6).

2. Major tumor-suppressor pathways

2.1. The RB pathway (CDK inhibitor–Cyc/CDK–RB)

The retinoblastoma gene (*RB1*) is the first identified tumor suppressor gene [8]. Individuals with heterozygous deletion or mutation of the *RB1* gene are susceptible to retinoblastoma in early life by additional deletion or mutation of the other allele. The *RB1* gene product pRB, together with its relatives, p107 and p130, comprises a family of pocket proteins [9, 10] (Hereafter we refer all pocket proteins to RB, and the *RB1* gene product to pRB). The main target of RB is the transcription factor E2F, which plays central role in cell proliferation by activating growth-promoting genes including those required for DNA replication and cell cycle progression [9]. In quiescent phase, RB binds to E2F and suppresses transcriptional activity of E2F. Moreover, RB recruits various chromatin-modifying factors, such as histone deacetylases (HDACs) [11] and histone methyl transferase SUV39H1 [12] to actively suppress expression of E2F target genes. Hence the main role of RB in tumor suppression is thought to be suppression of cell proliferation through suppression of E2F target gene expression. Growth stimulation induces expression of cyclins and activates cyclin dependent kinases (CDKs), which are called accelerators and engines in cell cycle progression, respectively. CDKs, in turn, inactivate RB by phosphorylation,

leading to expression of E2F target genes by releasing them from suppression by RB during G1 to S phase cell cycle progression (Figure 2) [13]. In contrast, growth-suppressive signals such as contact inhibition and DNA damage induce expression of CDK inhibitors, which are called brakes in cell cycle progression owing to their ability to inhibit activity of CDKs. Suppression of CDKs keeps RB in hypo-phosphorylated form, which binds to and inhibits E2F. Consequently, the activity of E2F to activate growth-promoting genes is controlled by the activity of RB, which is regulated by CDKs and CDK inhibitors. The pathway converging to RB, including CDKs and CDK inhibitors, is referred to the RB pathway.

Whether a cell progresses one round of the cell cycle or not is determined at the restriction point, which is located at late G1 phase of the cell cycle. Once a cell passed through the restriction point, it is programmed that the cell cycle automatically proceeds to the end of M phase. Thus, whether a cell proliferates or not is determined by whether the cell passes through the restriction point or not. Two major determinants whether a cell passes through the restriction point or not are E2F activity and cyclin dependent kinase activity, which is induced by E2F through activation of the *Cyclin E* (*CycE*) gene [14]. Since E2F plays essential roles in passing through the restriction point, RB can be regarded as a gatekeeper in cell cycle progression by controlling E2F activity. Hence disruption of the RB pathway and consequent activation of E2F is thought to be an essential event for tumorigenesis [2]. Actually, deletion or mutation of the *RB1* gene is observed in about 30% of cancers. Moreover, defects in the RB pathway upstream of RB such as overexpression of CycD and dysfunction of CDK inhibitors such as p16[INK4a] are frequently observed in other cancers retaining *RB1* [2]. It is predicted that all cancers have at least some defect in the RB pathway.

The RB pathway plays essential roles in tumor suppression by inducing cell cycle arrest or cellular senescence through suppression of the activity of E2F. As described below, disruption of the RB pathway reinforces cell cycle arrest by induction of CDK inhibitor p21[Cip1] expression through the p53 pathway or p27[Kip1] expression. When cells failed to induce cell cycle arrest, apoptosis is triggered through the p53 pathway or TAp73, a p53 family member, which activates various pro-apoptotic genes.
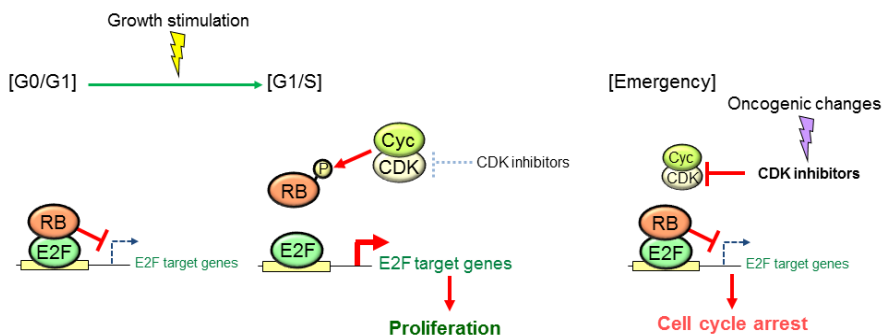
Figure 2. The regulatory mechanism of the transcription factor E2F by the RB pathway.

2.2. The p53 pathway (ARF–p53–cell cycle arrest or apoptosis related effectors)

The tumor suppressor p53 is a transcription factor that is activated by a variety of stress sig-
nals, including DNA damage, hypoxia and various oncogenic changes including aberrant
activation of E2F [15]. In response to such stress signals, p53 induces either cell cycle arrest
or apoptosis. Cell cycle arrest is mainly mediated through activation of the CDK inhibitor
$p21^{Cip1}$ gene [16], whose product suppresses wide range of CDKs. Apoptosis is mainly medi-
ated through activation of the *Bax* and BH3 only family genes, whose products destabilize
mitochondrial membrane to facilitate cytochrome c release, which triggers apoptotic cas-
cades of caspase activation.

Since p53 plays crucial roles in induction of cell cycle arrest or apoptosis, the expression
level of p53 is kept low by rapid ubiquitin/proteasome-dependent degradation, mainly
caused by Mdm2 (mouse double minute 2, Hdm2 in humans), which is often overex-
pressed in many cancers [15]. Mdm2 is E3 ubiquitin ligase, which directly binds to p53
and promotes p53 degradation. Mdm2 also inhibits *TP53* mRNA translation [17]. The
Mdm2 gene is a target of p53, forming a negative-feedback loop to control the level of
p53 [18]. Another negative regulator of p53 is MdmX, also known as Mdm4. MdmX has
recently emerged as a discrete critical negative regulator of p53 [19]. Though MdmX is
not a direct target of p53, structure of MdmX is significantly similar to that of Mdm2.
MdmX is reported to enhance p53 ubiquitination by altering the substrate preference of
the Mdm2, thereby indirectly regulating p53 [20].

Regarding response to oncogenic stresses, a potent activator of p53 is the tumor suppressor
ARF (alternative reading frame, known as p14 in humans and as p19 in rodents) [2]. ARF
directly binds to and sequesters Mdm2 to nucleoli, stabilizing p53 that leads to the expres-
sion of its target genes [21]. The pathway including upstream and downstream of p53 is re-
ferred to the p53 pathway. Of note, the *ARF* gene is a direct target of E2F [22] and this E2F-
ARF interaction connects the RB pathway and the p53 pathway, enabling efficient tumor-
suppressive response. When the RB pathway is disrupted by oncogenic changes, E2F is
activated to induce ARF gene expression, leading to activation of the p53 pathway, which
inhibits oncogenic cell growth by inducing cell cycle arrest or apoptosis (Figure 3). There-
fore, disruption of both of the tumor suppressor pathways powerfully shifts the cell-fate de-
termination balance to proliferation (Figure 1) and is thought to be essential to induce
deregulated cell proliferation that leads to tumorigenesis. Indeed, about 50% of cancers car-
ry *TP53* mutations or deletion and most cancers have defects in the p53 pathway including
those in upstream and downstream of p53.

2.3. The TAp73 pathway (E2F–TAp73–pro-apoptotic targets)

The tumor suppressor TAp73 is a homologue of p53 and can induce apoptosis independent-
ly of p53 [3, 4, 23]. The *TP73* gene encodes two isoforms, TAp73 and DNp73, which are driv-
en by different promoters. DNp73 lacks the transactivation (TA) domain and counteracts
TAp73 and p53 [24]. Thus DNp73 is anti-apoptotic. The *TAp73* gene is thought to be a tumor
suppressor gene and is known to be a target of E2F1 [3, 4, 25]. Similarly to p53, TAp73 is
activated by both oncogenic changes and DNA damage [26, 27] and TAp73 target genes

partly overlap with those of p53, such as the *PUMA* and *NOXA* genes [28, 29] that are crucial for induction of apoptosis. Moreover, TAp73 can induce apoptosis in the absence of p53 [3, 4]. Therefore, TAp73 seems to back-up the important tumor suppressive function of p53.
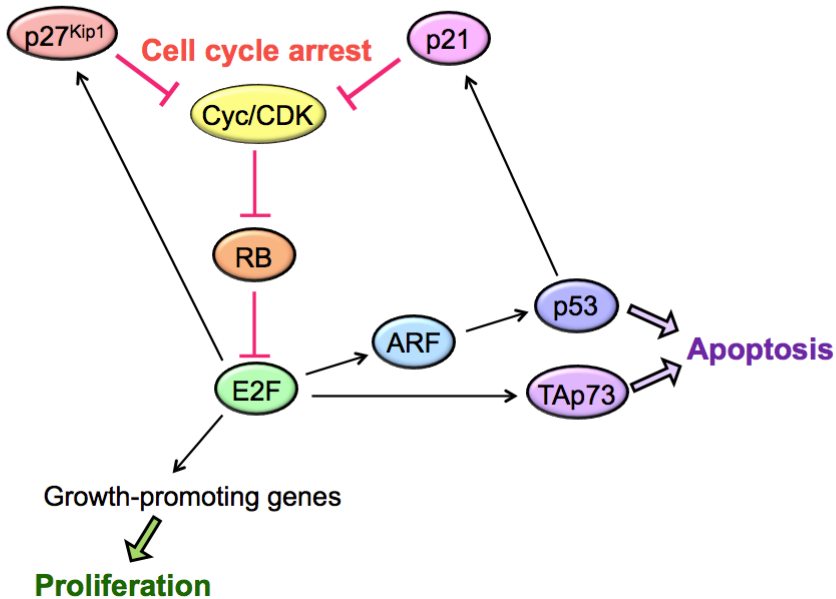
Figure 3. E2F activates major intrinsic tumor suppressor pathways.

3. The transcription factor E2F

The transcription factor E2F was originally identified as a cellular DNA-binding protein, which mediate E1A-dependent activation of the adenovirus E2 promoter [30]. Members of E2F family are downstream targets of the tumor suppressor RB and make repressor complexes with RB that keep cells in quiescent state [9, 10]. Though E2F plays central roles in cell proliferation by activating growth-promoting genes, certain members of E2F can induce apoptosis [15]. In this paragraph, we will describe three points as follows (1) E2F family members, the role of them in cell cycle progression, cell cycle arrest and apoptosis. (2) Regulatory mechanism of E2F. (3) E2F target genes, to better understand the regulatory mechanism of cell-fate determination mediated through E2F.

3.1. E2F family members

E2F consists of eight family members (E2F1-E2F8). E2F1-E2F5 are bound by their repressor RB family proteins through RB binding domain. E2F1-E2F5 activate transcription when free

from RB and repress transcription when bound by RB. E2F1-E2F3a are induced at G1/S boundary and activate transcription free from RB. In contrast, E2F3b-E2F5 are expressed all through the cell cycle and play main roles in transcriptional repression in G0/G1 bound by RB. E2F6-E2F8 repress transcription independently of RB. Hence E2F family members are divided into two groups: activator E2Fs (E2F1-E2F3a) and repressor E2Fs (E2F3b-E2F8). Activator E2Fs play major roles in activation of target genes involved in growth promotion, growth suppression and induction of apoptosis. Repressor E2Fs are roughly divided into two groups; E2F3b-E2F5, which make repressor complex together with RB, and recently identified E2F6-E2F8, which function independently of RB.
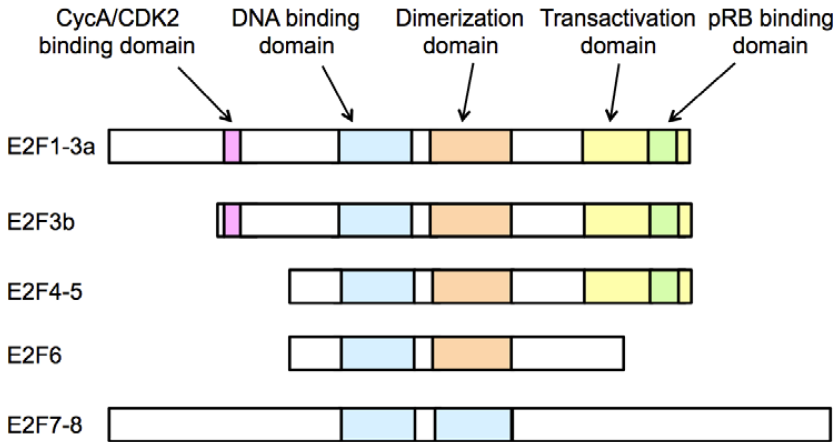
Figure 4. Structure of E2F family members. E2F1-E2F6 bind to their target promoter with binding partner DP proteins through dimerization domain. E2F7 and E2F8 have two DNA binding domains and make homodimers or E2F7/E2F8 heterodimer.

A structural characteristic of activator E2Fs (E2F1-E2F3a) is longer N terminal region, which does not exist in repressor E2Fs (E2F3b-E2F6). Expression of E2F1-E2F3a is induced by E2Fs themselves [31]. When growth stimulation inactivates p130 by phosphorylation through activation of CycD/Cdk4 or CycD/Cdk6, repressor E2Fs (E2F4 and E2F5) are released from p130 and activates the *E2F1-E2F3a* genes. E2F1-E2F3a in turn replace E2F4 and E2F5, and activate growth-promoting genes including E2F1-E2F3a themselves. Each activator E2F has preferential roles in cell cycle progression and induction of apoptosis. p107 preferentially make complexes with E2F4-E2F5 in G1/S to S phases.

E2F1 is generally thought to be the most powerful transcriptional activator of pro-apoptotic genes among activator E2Fs. Overexpression of E2F1 in tissue culture cells alone can induce cell cycle progression in otherwise quiescent fibroblasts [32]. Overexpression of E2F1 can be oncogenic *in vitro* [33, 34] and *in vivo* [6]. In contrast, E2F1 is dispensable for cell cycle progression, since E2F1 knockout mice are viable. On the other hand, over expression of E2F1 in cancer cell lines leads to apoptosis [35]. E2F1 also plays a key role in induction of cellular

senescence by activating the ARF-p53 pathway, when overexpressed in human normal fibroblasts [36]. Moreover, E2F1 null mice resulted in tumorigenesis [7]. Taken together, E2F1 seems to play the most important roles in tumor suppression among activator E2Fs by activating genes involved in apoptosis and cell cycle arrest.

E2F2 has 46% overall amino acid sequence similarity to E2F1 [37] and is thought to play roles in both cell proliferation and tumor suppression. E2F2 null mice exhibit increased proliferation of hematopoietic cells and frequently develop autoimmunity and tumors [38, 39]. In tumor suppression, E2F2 plays major roles in suppression of Myc-induced T cell lymphomagenesis. Inactivation of neither E2F1 nor E2F3 had no effect on tumor progression in T cells, and only loss of E2F2 accelerated lymphomagenesis [40].

E2F3 is thought to be the most important activator E2F in cell proliferation. Although E2F1 or E2F2 null mice are viable and tumor-prone, E2F3 null mice are typically embryonic lethal in pure background [41] or show partially penetrant embryonic lethality in mixed background [42]. Although mouse embryonic fibroblasts (MEFs) with combined knockout of E2F1 and E2F2 proliferate, those with combined knockout of E2F1, E2F2 and E2F3 fail to proliferate and can not re-enter the cell cycle [5]. The E2F3 locus encodes two isoforms, E2F3a and E2F3b, a truncated variant of E2F3 in its N-terminus [43, 44]. Although both E2F3s partly overlaps their roles in cell cycle progression [45], E2F3a is expressed in G1/S to S phases and is thought to play crucial roles on cell proliferation, while E2F3b is expressed equivalently in quiescent and proliferating cells, and associates with pRB, representing the predominant E2F-pRB complex in quiescent cells [44, 46]. E2F3b also plays roles in myogenic differentiation by promoting gene expression related to differentiation [47].
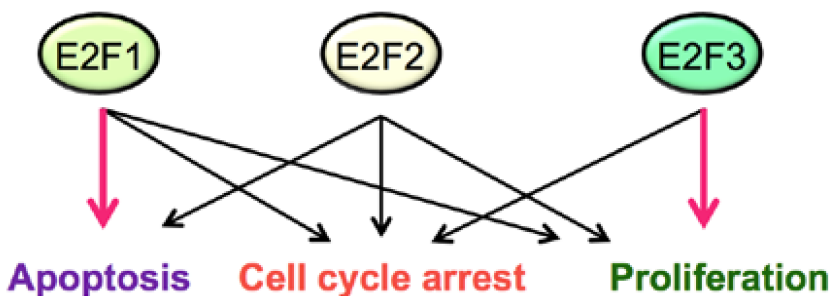
Figure 5. Roles of activator E2Fs in cell-fate determination. E2F1 plays crucial roles in induction of apoptosis. E2F3 is thought be essential for cell proliferation. It is predicted that the character of E2F2 is in the middle of E2F1 and E2F3.

E2F4 and E2F5 were cloned by their association with p107 and p130, and are significantly detected in quiescent cells [48, 49]. Knockout mice of either *E2F4* or E2F5 are viable [50-52]. E2F4 knockout mice are runted and display defects in late stage of maturation. In addition, these mice present reduced thickness of the gut epithelium and developmental craniofacial defects. E2F5 knockout mice develop hydrocephalus after birth apparently due to increased secretion of cerebrospinal fluid by the choroid plexus. E2F4 and E2F5 double knockout mice die before birth because of developmental defects, suggesting that E2F4 and E2F5 have some redundant functions during development [53]. Cells lacking E2F4 and E2F5 are unable to stop cell cycling upon growth-suppressive signals such as TGF-β treatment, suggesting that E2F4 and E2F5 are major repressor E2F in restraining cell cycle progression. These two E2Fs are thought to be important for cell cycle exit and terminal differentiation.

E2F6 does not possess RB binding domain. As predicted from the structure, E2F6 contributes gene silencing independent of RB [54] and is predicted to make repressor complex with polycomb proteins [55]. Consistent with this, *E2f6-/-* animals display overt homeotic transformations of the axial skeleton that are strikingly similar to the skeletal transformations observed in polycomb deficient mice [56]. E2F6 is reported to bind the same E2F-repressor site as E2F4, suggesting E2F6 may partly overlaps its function as transcriptional repressor with E2F4 in S phase [57].

E2F7 and E2F8 are thought to function as transcriptional repressors independently of RB proteins. An important target of E2F7 and E2F8 is the *E2F1* gene. E2F7 and E2F8 knockout mice are embryonic lethal, at least in part, due to apoptosis caused by inability to down regulate expression of E2F1, leading to activation of p53 [58]. Recent report showed that E2F7 and E2F8 promote angiogenesis through transcriptional activation of the VEGFA promoter with hypoxia inducible factor 1 (HIF1) [59].

3.2. Regulatory mechanism of E2F

E2F1 through E2F6 associate with DP family proteins (DP1 or DP2) to form heterodimeric complexes that bind to DNA in a sequence-specific manner (consensus sequence: $TTT^C/_G{}^G/_CCGC$). E2F7 and E2F8 have two DNA binding domains and do not require DP proteins for binding to DNA. E2F7 and E2F8 make homodimer or E2F7/E2F8 heterodimer to bind to the target [58].

Transcriptional activity of E2F1 through E2F5 is suppressed by binding of RB family proteins. pRB preferentially binds E2F1 through E2F3, whereas p107 and p130 preferentially bind E2F4 and E2F5. However, pRB can also bind E2F4, depending on the cellular circumstances. E2F6 through E2F8 can repress transcription independently of pRB family proteins [10, 60]. In quiescent phase, E2F3b/pRB, E2F4/p130 and E2F5/p130 repress promoters of E2F target genes. Upon growth stimulation, activated CycD/Cdk4 and CycD/Cdk6 phosphorylate pRB and p130, inhibiting their binding to the E2Fs and allowing accumulation of the free E2Fs [31]. This release from repression conferred by the pRB family proteins is the primary activation step for induction of E2F target genes. In this context, E2F3b, E2F4 and E2F5 mainly act as repressors together with the pRB family proteins during G0/G1 phases. Expression of E2F1, E2F2 and E2F3a is induced at the G1/S boundary by E2F itself, and activate

the growth related genes, including the *CycE* gene. CycE activates Cdk2, whose activity is essential for initiating DNA replication, and drive cells into S phase [31]. In late S phase, CycA/Cdk2 complex represses the transcriptional activity of E2F/DP complex by phosphorylation, which releases the complex from the binding element [61].

3.3. E2F target genes

Classical E2F targets are genes involved in DNA replication and cell cycle progression. In addition to these, recent studies with DNA microarray and chromatin immunoprecipitation (ChIP) identified a variety of E2F targets. These include genes involved in DNA repair, checkpoint, differentiation, development, metabolism, micro RNAs, apoptosis, cell cycle arrest and others.

DNA replication

E2F regulates expression of most of the genes involved in initiation of DNA replication: the *ORC1* (*origin recognition complex1*), *CDC6*, *MCM* (*maintenance of minichromosome*) *2-7*, *ASK* and *CDC45* genes. These factors are assembled into pre-replication complex, which is activated by CycE/Cdk2 to initiate DNA replication [31]. Cdt1 also plays crucial roles in initiation of DNA replication and is negatively regulated by geminin. The *Cdt1* and *geminin* genes are both E2F targets [62]. Genes, which code for machineries responsible for DNA replication, are classical E2F targets. These include the *DHFR* (*dihydrofolate reductase*), *DNA polymerase α*, *thymidine kinase, thymidylate synthase* and *PCNA (proliferating cell nuclear antigen)* [53].

DNA repair, checkpoint

E2F target genes related to DNA repair are the *Rad51*, *MSH2* and *MLH1* genes, which are involved in homologous recombination repair and mismatch repair [63]. E2F target genes related to checkpoint are the *ATM*, *Chk1*, *Mad3*, *Bub1*, *Claspin* and *RanBP1* genes [53, 64-67]. It is expected that E2F regulates these DNA repair and checkpoint genes to prepare machineries to quickly respond in case of emergency.

Cell cycle progression

E2F induces expression of genes, which play major roles in induction of S phase, such as the *CycE* and *activator E2F* (*E2F1-E2F3a*) genes themselves [31]. Also, the upstream negative regulators of pRB, the *Emi1* and *Skp2* genes are E2F targets [68, 69]. The genes, which encode CycA, Cdc2 (CDK1), CycB and B-myb that are important for S and G2/M phase progression, are also E2F targets [53]. Repressor E2Fs, *E2F7* and *E2F8* genes are reported to be targets of E2F1 that promote embryonic development by suppressing E2F1-p53 induced apoptosis, forming a negative feedback loop. Although c-Myc is reported as a target of E2F [70], E2F may play a role in suppression of c-Myc expression upon negative growth signals [71].

Development and differentiation

E2F also regulates expression of genes involved in development and differentiation. The *Firizzled homologs1-3*, *Homeobox* and *TGF* genes are shown to be targets of E2F

[53]. It is reported that overexpression of E2F1-3 induced various genes involved in development and differentiation [72]. Interestingly, although E2F7 and E2F8 are generally regarded as transcriptional repressors, a recent study reported the roles of E2F7 and E2F8 in transcriptional activation of genes such as VEGFA as written before [59].

Cellular metabolism

Recent work reported that E2F1 and pRB are required for repression of genes implicated in oxidative metabolism [73]. E2F1 repressed key genes that regulated energy homeostasis and mitochondrial functions in muscle and brown adipose tissue, and E2F1 null mice had a marked oxidative phenotype. Their work suggests a metabolic switch from oxidative to glycolytic metabolism that responds to stressful conditions.

Micro RNAs

Accumulating evidence indicates that expression of E2F is regulated by microRNAs and that E2F also induces expression of microRNAs [74]. One of the major microRNAs regulated by E2F is miR-17~92. miR-17~92 is a negative regulator of E2F1-E2F3 and is also a target of E2F1-E2F3, constructing a fail-safe mechanism to regulate E2F activity. E2F seems to be rigorously controlled by many miRNAs, suggesting that E2F activity must be strictly controlled for appropriate cell cycle progression.

Apoptosis and cell cycle arrest

E2F can activate genes involved in apoptosis and cell cycle arrest, which are inconvenient for cell proliferation. Regulatory mechanism of these growth-suppressive genes by E2F is yet to be elucidated, especially regarding regulation of cell growth versus tumor suppression in response to normal growth stimulation and oncogenic changes. The *caspase3*, *caspase7* and *Apaf1* genes, which code for apoptotic machineries, are direct targets of E2F [53]. Caspases are expressed as inactive precursors (procaspases), and expression of procaspases and Apaf-1 alone does not necessarily induce apoptosis. These apoptotic machineries require upstream signals to be activated to induce apoptosis. Expression of these two pro-apoptotic E2F targets and all E2F targets mentioned above is induced by growth stimulation. Thus, expression of the pro-apoptotic machinery by growth stimulation is thought to be fail-safe mechanism to induce apoptosis in case of emergency. We refer these E2F target genes, whose expression is induced by growth stimulation, to 'typical E2F targets'. Typical E2F targets are activated by growth stimulation, which physiologically inactivates RB by phosphorylation and activates E2F by release from repression.

In contrast to typical E2F targets, we identified three E2F targets, which are not activated by growth stimulation. These include the tumor suppressor *ARF*, *TAp73* and CDK inhibitor *p27^{Kip1}* genes. As described below, these genes are critically important in tumor suppression. We describe the regulatory mechanism of the tumor suppressor *ARF*, *TAp73* and CDK inhibitor *p27^{Kip1}* genes by E2F in next paragraph.
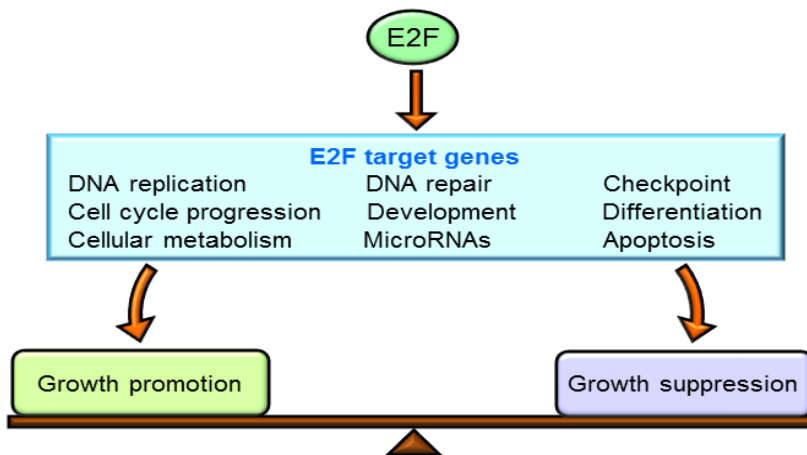
Figure 6. E2F activates both growth-promoting genes and growth- suppressive genes, including E2F itself. It is predicted that the expression levels of E2F targets, related to growth- promotion or growth- suppression, decide the balance of cell-fate determination.

4. Deregulated E2F

Since E2F1-E2F3a are activated by growth stimulation, it is generally thought that their target genes are all activated by growth stimulation. However, E2F1-E2F3a also activate genes involved in cell cycle arrest or apoptotic, which are inconvenient for cell growth. It has yet to be elucidated how E2F regulates genes involved in cell cycle arrest or apoptosis, regarding normal cell growth and tumor suppression.

Since all E2F targets are thought to be activated by growth stimulation, it is surprising that our previous work identified three growth suppressive E2F targets, which were not activated by growth stimulation at all in human normal fibroblasts [75-77]. In contrast, these E2F targets were activated by deregulated E2F activity induced by overexpression of E2F1 or forced inactivation of pRB. Overexpression of E2F1 generates exceeding amount of exogenously introduced E2F1, which becomes out control by RB proteins. Forced inactivation of pRB induces endogenous deregulated E2F activity out control by pRB. We refer these growth suppressive E2F targets, which are not activated by growth stimulation but are activated by deregulated E2F, to 'atypical E2F targets'. These atypical E2F targets include the tumor suppressor *ARF* and *TAp73* genes and the CDK inhibitor *p27^{Kip1}* gene These three atypical E2F target genes play major roles in tumor suppression. ARF is an upstream activator of the tumor suppressor p53. CDK inhibitor p27^{Kip1} activates the RB pathway by inhibiting CDKs. TAp73 is the tumor suppressor, which can induce apoptosis in dependently of p53. Our observations suggest that E2F activity induced by RB dysfunction, one of major on-

cogenic changes, has distinct function from that induced by growth stimulation in activating target genes (Figure 7).

E2F activity induced by RB dysfunction activates both typical and atypical E2F targets. In contrast, E2F activity induced by growth stimulation activates only typical E2F targets and interestingly, not atypical E2F targets. Both of the E2Fs are similar in the sense that they are released from repression by RB. However, it is shown that growth stimulation does not totally inactivate pRB and some portion of activator E2Fs is still in complex with pRB [78]. Moreover, the *RB1* gene is an E2F target [79] and expression of pRB is increased in G1/S to S phases [80]. Indeed, the amount of activator E2Fs/pRB complex rather increases in G1/S to S phases, as examined by gel mobility shift assay [78]. This observation indicates that E2F activity induced by growth stimulation is still under control of RB. In contrast, E2F activity induced by dysfunction of RB is thought to be out of control by RB. Here, we refer E2F activated by growth stimulation to 'physiological E2F' and that by dysfunction of RB to 'deregulated E2F'. Our findings indicate that deregulated E2F is functionally different from physiological E2F.

In this paragraph, we describe the regulatory mechanism of atypical E2F targets by deregulated E2F and discuss about the characteristics of deregulated E2F activity regarding its role in tumor suppression.
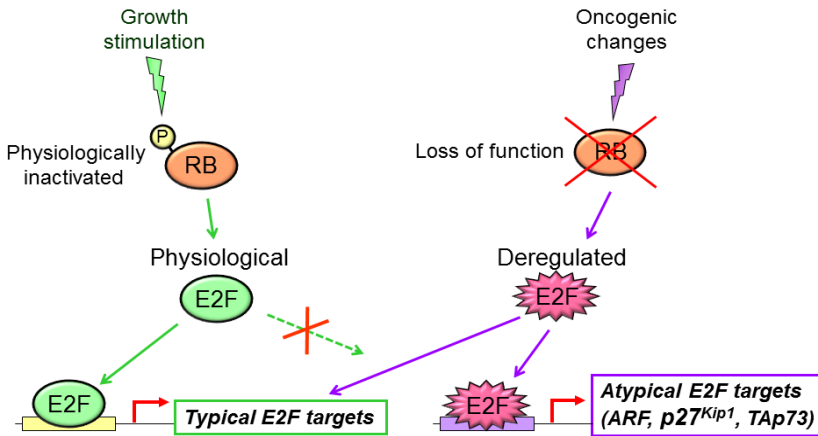
Figure 7. Atypical E2F target genes are specifically activated by deregulated E2F activity through specific E2F responsive elements.

4.1. Atypical E2F targets

The first atypical E2F target identified was the *ARF* gene [75]. ARF is the major activator of p53 pathway and links the RB and p53 tumor suppressor pathways [22], playing crucial roles in tumor suppression. Consistent with this notion, deletion, mutation or silencing of

the *ARF* gene is frequently observed in cancers. Moreover, *Arf* null mice are highly prone to tumorigenesis [81]. Various oncogenic signals are able to elicit the activation of the *ARF* gene. Overexpression of adenovirus E1a or E2F1 in primary mouse embryonic fibroblasts (MEFs) rapidly induces ARF gene expression and p53-dependent apoptosis [16]. Myc over-expression and oncogenic mutant Ras are also strong activators of the *ARF* gene and combination of absence of Arf in mice severely impaired the tumor suppressive activity of p53 [82]. Arf promoter seems to monitor these oncogenic signals as shown by ARF promoter-*GFP* transgenic model, in which GFP expression was observed in tumors induced by Myc or Ras but not in normal growing tissues [83]. Other studies elucidated that ARF also restrains cell growth independently of p53, interacting with other factors [84]. Taken together, the *ARF* gene plays crucial roles in tumor suppression through p53-dependent and independent pathways.

The CDK inhibitor p27^{Kip1} is an upstream regulator of the RB pathway and known to contribute to the ability of pRB to induce cell-cycle arrest, differentiation and senescence [85-87]. There is cross regulation between p27^{Kip1} and pRB. p27^{Kip1} enhances pRB growth suppressive function by inhibiting Cyc/CDK, keeping pRB in hypo-phosphorylated form. pRB increases the amount of p27^{Kip1} by sequestering Skp2, a component of E3 ubiquitin ligase complex, which promotes degradation of p27^{Kip1} [86]. pRB also known to cooperate with APC/C^{cdh1}, another E3 ubiquitin ligase, to induces Skp2 degradation, stabilizing p27^{Kip1} [88]. Taken together, pRB and p27^{Kip1} seem to keep close relationship in the RB pathway to efficiently suppress aberrant cell proliferation. Indeed, our previous study showed that inactivation of RB by adenovirus E1a increased BrdU (bromodeoxyuridine)-positive cells much earlier in *p27^{Kip1}*-/- MEFs than in wild type MEFs [66]. These results support the notion that p27^{Kip1} plays important roles in the RB pathway to suppress cell cycle progression induced by oncogenic changes.

The tumor suppressor *TAp73* gene had been identified as a direct target of E2F using cancer cell lines [4]. We found that the *TAp73* gene was activated by deregulated E2F but not by physiological E2F in human normal fibroblasts [77]. TAp73 is a p53 family member and plays important roles in tumor suppression with its other family member p53 and p63. All of the three genes express differentially spliced isoforms [89, 90]. Two major isoforms are TA isoforms, which retain transactivation (TA) domain, and delta N (DN) isoforms, which lack TA domain. Since these family members activate their targets as tetramers and DN isoforms lack transactivation (TA) domain, DN isoforms have dominant-negative properties [24]. Although p53 is deleted or mutated in half of all human cancers, deletion or mutations of p73 and p63 occur rarely [91, 92]. Rather over expression of DN form of p73 and p63 are commonly observed in many cancers, such as over expression of DNp73 isoform in gliomas and carcinomas of the breast and the colon [93, 94], and that of DNp63 isoform in bladder carcinomas [95]. Each DN isoform can suppress all three types of TA forms [92, 96, 97]. Therefore, it is expected that tumor-suppressive TA isoforms are suppressed by DN isoforms in many cancers. This could explain why deletion or mutations of the *TP73* and *TP63* genes are rare. *TAp73* can induce apoptosis independently of p53. Moreover, TAp73 knockout mice are tumor prone,

infertile, sensitive for carcinogen-induced tumorigenesis and defective for maintenance of genomic stability [98]. The *TAp73* gene is activated by various stress signals including deregulated E2F and DNA damage. These observations suggest that TAp73 contributes to tumor suppression in addition to the p53 pathway in response to various oncogenic changes.

Accumulating evidence indicates that the three atypical E2F targets explained above play crucial roles in tumor suppression, by activating the RB pathway, the p53 pathway and the p53 independent pathway. Taken together, deregulated E2F seems to be critically important for activating major intrinsic tumor-suppressor pathways in responding to oncogenic changes to suppress tumorigenesis (Figure 8).
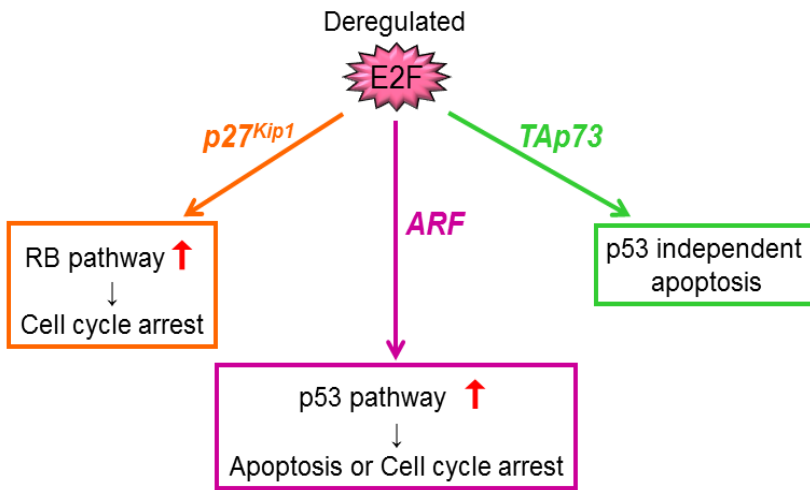
Figure 8. Deregulated E2F plays important roles in activating major tumor suppressor pathways (RB, p53 and TAp73 pathways) by activating the *ARF*, *p27^Kip1* and *TAp73* genes.

4.2. Distinct transcriptional regulatory mechanism mediated by deregulated E2F

ARF, p27^{Kip1} and TAp73 exert its effects when expressed unlike pro-apoptotic targets, which are expressed as inactive precursors such as pro-caspases. Thus the regulation of expression of these genes is critically important for tumor suppression. The finding that these genes are specifically activated by deregulated E2F but not by physiologically activated E2F indicates that there is a mechanism to specifically respond to oncogenic changes to suppress tumori-genesis, while allowing normal cell growth upon normal growth stimulation.

We first identified the tumor suppressor *ARF* gene as an atypical E2F target [75]. In our studies, we used human normal fibroblasts (HFFs or WI-38) to examine the responsiveness of each growth-suppressive E2F target to physiological and deregulated E2F. This is because most of previous studies used cancer cell lines and were unable to examine responsiveness

of E2F target genes to normal growth stimulation. To induce physiological E2F, we used serum stimulation, common growth stimulation for fibroblasts. To induce deregulated E2F activity, we used ectopically expressed E2F1 or forced inactivation of RB either by adenovirus E1a, which binds to and inactivates all RB family proteins, or shRNA against *RB1* (shRB), which represses the expression of pRB. The latter (adenovirus E1a and shRB) is expected to induce endogenous deregulated E2F activity.

Ectopically expressed E2F1, adenovirus E1a and shRB induced ARF gene expression in RT-PCR, and activated ARF promoter in reporter assay. However, serum stimulation, which physiologically activates E2F, did not induce ARF gene expression or activate ARF promoter under the condition that CDC6 gene (one of typical E2F targets involved in DNA replication) expression was significantly induced and CDC6 promoter was clearly activated. These results indicate that the *ARF* gene is specifically activated by deregulated E2F but not by physiological E2F. Promoter analyses identified the E2F responsive element of ARF promoter (EREA), which specifically responds to deregulated E2F activity. Interestingly, the sequence of EREA was composed of only GC repeat and lacked T stretch. This is in contrast to that of consensus E2F binding motif, which is composed of T stretch and GC repeat ($TTT^G/_C{}^C/_GCGC$) in typical E2F targets. In addition, the location of EREA was far upstream from transcription start site compared to that of typical E2F sites, which is within 100 bp from transcription start site in most cases. Moreover, our gel mobility shift assay and ChIP assay showed that EREA specifically binds ectopically expressed E2F1 but not physiological E2F1 induced by serum stimulation, both *in vitro* and *in vivo*, respectively. Taken together, these observations suggest that the *ARF* gene is specifically activated through EREA by deregulated E2F activity, triggered by ectopically expressed E2F1 or forced inactivation of RB, but not by physiological E2F activity, induced by serum stimulation (Figure 7).

A later study showed that ARF promoter lacking EREA was still activated by overexpression of E2F1 [99]. Our further analyses of ARF promoter identified multiples of EREA-like elements in ARF promoter. It seems that, although EREA is the major E2F responsive element in ARF promoter, it is not the sole responsive element and multiple EREA-like elements co-operate to specifically respond to deregulated E2F activity (manuscript in preparation).

Defects in the RB pathway activate E2F out of control by RB, promoting abnormal cell growth. In response to such oncogenic insults, deregulated E2F activates the *ARF* gene, leading to activation of p53 to protect cell from tumorigenesis. For induction of tumorigenesis, the p53 pathway must be disabled by further oncogenic changes. Indeed, defects in the p53 pathway are observed in almost all cancers. It is expected that the presence of deregulated E2F be tolerated in cancer cells by further inactivation of the p53 pathway. We thus examined the existence of deregulated E2F activity in *RB1* deficient cancer cell lines and in normal growing fibroblasts. When we introduced constitutively active form of pRB into *RB1* deficient cancer cell lines (5637, Saos-2 and C-33 A) and normal growing fibroblasts (WI-38 and HFF), activity of EREA and ARF promoter were decreased in *RB1* deficient cancer cell lines, but not in normal growing fibroblasts. These results showed that deregulated E2F activity specifically exists in *RB1* deficient cancer cell lines but not in normal growing fibro-

blasts. The presence of deregulated E2F activity may serve as a useful marker to discriminate cancer cells from normal growing cells.

Our search for new E2F targets with subtraction method identified the CDK inhibitor *p27^{Kip1}* gene as an atypical E2F target [66]. p27^{Kip1} plays important roles in cell cycle arrest by inhibiting Cyc/CDKs. Using reporter assay, we showed that EREK (E2F responsive element of p27^{Kip1}) was responsible for specifically sensing deregulated E2F activity in human normal fibroblasts and that EREK was specifically activated in the *RB1* deficient cancer cell lines [76]. Consistent with EREA, the location of EREK was far upstream compared to typical E2F binding sites. Interestingly, the sequence of EREK contained T in addition to GC repeat and is rather similar to that of typical E2F binging site. However, EREK bound deregulated E2F1 but not physiological E2F1 in ChIP assay, showing that its character was similar to EREA. These results suggest that not only the sequence of E2F responsive elements, but also the sequence around the responsive elements may be important for discriminating deregulated E2F activity from physiological E2F activity. There is also a possibility that structure of the whole promoter might also affect the discrimination.

Third atypical E2F target is the tumor suppressor *TAp73* gene [77]. TAp73 promoter specifically responded to deregulated E2F activity through four ERE73s (E2F responsive elements of TAp73), which were specifically activated by deregulated E2F activity. The sequences of ERE73s contained T stretch and were similar to that of typical E2F binding sites. Importantly, our ChIP assay showed that bindings of ectopically expressed 'exogenous' E2F1 and deregulated 'endogenous' E2F1 induced by adenovirus E1a were detected on ERE73s, but not that of physiological E2F1 induced by serum stimulation. Thus, although the sequences of ERE73s were similar to or almost same as that of typical E2F binding sites, the characters of both were completely different. ERE73s were specifically activated by deregulated E2F activity and specifically bound to both 'exogenous' and 'endogenous' deregulated E2F1. These results support the notion that both sequences of the E2F binding site and its flanking region may be important for discriminating deregulated E2F activity from physiological E2F activity. Consistent with EREA and EREK, reporter assay showed that ERE73s were also activated in the *RB1* deficient cancer cell lines and not in normal fibroblasts. Moreover, reintroduction of the constitutive active from of pRB by recombinant adenovirus reduced the expression of the *TAp73* gene in all the cancer cell lines in RT-PCR, indicating that the cancer cell lines harbor deregulated E2F activity that activates the endogenous *TAp73* gene.

Interestingly, our unpublished data suggest that not only the *RB1* deficient cancer cell lines but also cancer cell lines retaining pRB harbor deregulated E2F activity. Activity of ERE73s and expression of the *TAp73* gene were suppressed by introduction of the constitutive active form of pRB in cancer cell lines retaining pRB. These results suggest the possibility that E2F-mediated transcriptional program can sense defects in the RB pathway, not only pRB itself but also upstream regulators of pRB. Taken together, deregulated E2F activity might become a universal means to discriminate cancer cells (may be regardless of the presence of pRB) from normal growing cells.

4.3. Difference between deregulated E2F and physiologically activated E2F

Deregulated E2F and physiologically activated E2F are similar in a sense that both are 'released from RB'. However, there is a functional difference between deregulated E2F and physiologically activated E2F. Deregulated E2F activates both typical and atypical E2F targets. In contrast, physiologically activated E2F activates typical E2F targets but not atypical E2F targets. Why atypical E2F targets are activated by deregulated E2F and not by physiologically activated E2F? What is the difference between deregulated E2F and physiologically activated E2F? Deregulated E2F is totally 'out of control' by RB due to dysfunction of the RB pathway. In contrast, physiologically activated E2F is temporarily released from RB, predicted to be 'under control' of RB. During normal cell growth, activator E2Fs are induced at G1/S boundary of the cell cycle. At the point, it is generally believed that pRB is phosphorylated and inactivated. However, pRB is not totally inactivated at the point. Previous studies indicate that the *RB1* gene is an E2F target [79] and expression of pRB is increased in G1/S to S phases [80]. Moreover, the amount of activator E2Fs/pRB complex rather increases at G1/S boundary as shown by gel mobility shift assay [78], indicating that some portion of pRB is still active and is regulating the activity of activator E2Fs. Thus, physiologically activated E2F is still under control by pRB. It is likely that activity of activator E2Fs is strictly controlled by degree of phosphorylation of pRB dependent on the activity of CDKs, reflecting the strength of growth stimulation. There must be difference between activation of E2F out of control by pRB and activation of E2F under control by pRB. Our studies of regulatory mechanism of atypical target promoters by E2F elucidated the four different points between regulation of typical E2F target promoter and that of atypical E2F target promoter.

1. Although sequences of EREK and ERE73s resemble that of typical E2F binding sites, sequence of EREA is different from that of typical E2F sites. The sequence of consensus E2F binding motif of typical E2F targets is composed of T stretch and GC repeat (TTT$^G/_C$$^C/_G$CGC). EREA is composed of only GC repeat and lacks T stretch. Difference in binding sequence suggests the possibility that there may be a difference in factors, which recognize the sequence and bind.

2. In all three cases, location of E2F responsive elements of atypical E2F targets is far from the transcriptional start site compared to that of typical E2F binding sites in typical E2F targets. In the case of typical E2F targets, location of E2F binding sites is within 100 bp from transcriptional start site in most cases. Typical E2F targets are under repression by E2F/RB complex in quiescent phase and are released from repression upon growth stimulation. Close proximity of E2F binding sites to transcriptional start site may be required for this mode of regulation. In contrast, atypical E2F targets are not under repression by E2F/RB complex and literally activated by deregulated E2F. E2F responsive elements of atypical E2F targets behave as enhancer elements, which can function from distance.

3. Deregulated E2F1 bound to EREA, EREK and ERE73s and physiologically activated E2F1 did not bind to these elements as shown by ChIP assay. In the case of EREA, it is also shown that repressor type E2F4 does not bind to EREA. This observation is compatible with the observation that these atypical E2F targets are not under repression by

E2F/RB. The fact that deregulated E2F bind to atypical E2F targets, while physiological E2F does not bind to atypical E2F targets, suggest that there is difference in binding behavior between deregulated E2F and physiologically activated E2F.
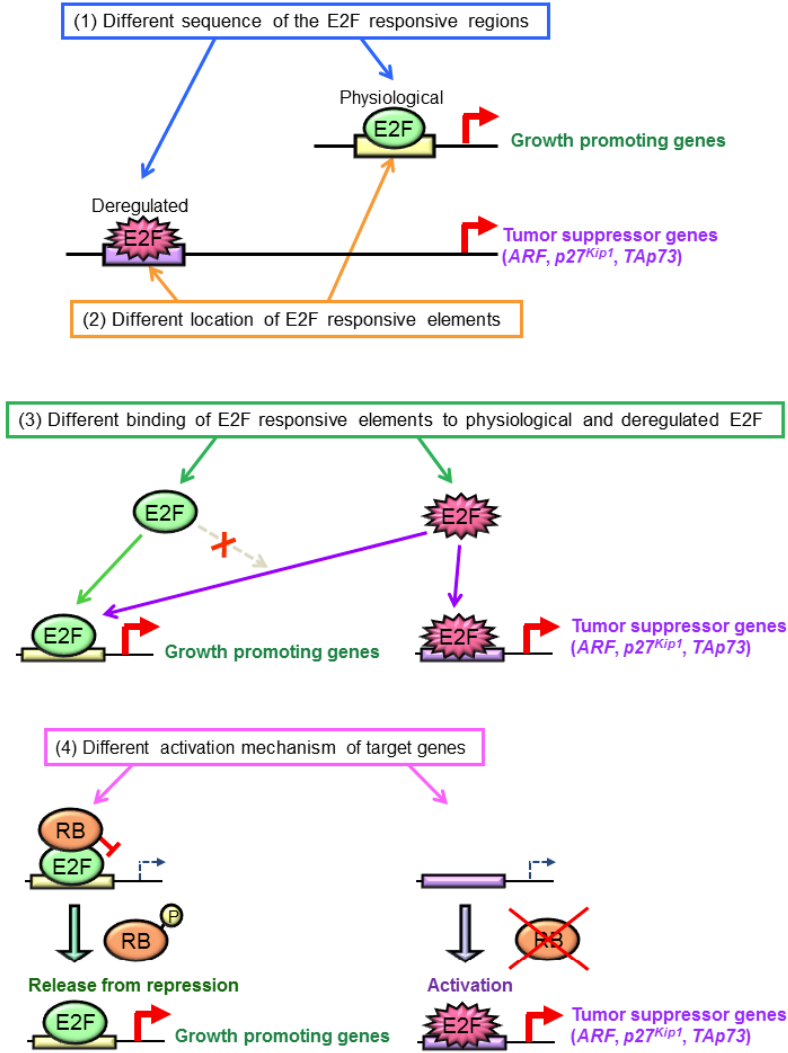
Figure 9. Differences in E2F regulation of target promoters between deregulated E2F and physiological E2F. (1) Sequence of the atypical E2F responsive elements and surrounding regions are different from that of typical E2F targets. (2) E2F responsive elements of the atypical E2F targets locate far upstream from transcriptional start sites compared to that of typical E2F targets. (3) E2F responsive elements of atypical E2F targets specifically bind deregulated E2F and not physiologically activated E2F. (4) Unlike typical E2F targets, promoters of atypical E2F targets are not under repression of RB and are specifically activated by deregulated E2F activity.

4. Regulatory mechanism of promoters is different between typical E2F targets and atypical E2F targets. Promoters of typical E2F targets are repressed by E2F/RB complex. Growth stimulation inactivates RB and releases promoters from the repression by RB. Thus, so-called activation of typical E2F targets by physiological E2F is 'release from repression by RB'. In contrast, activation of atypical E2F targets by deregulated E2F is literally 'activation'. Mutation of EREA, EREK or ERE73s in corresponding full-length promoter did not enhance basal promoter activities, indicating that these three promoters are not under repression through the E2F responsive elements. This is consistent with the observation that binding of physiological E2F to promoters of atypical E2F targets was not observed in ChIP assay, including repressor type E2F4 (Figure 9).

It is generally accepted that the amount of free E2F is important for differential regulation of E2F targets, which have opposite roles in cell-fate determination, as proposed as threshold model [100]. According to this model, when the amount of free E2F (released from repression of RB) is below the threshold, E2F activates only growth-related target genes. When the amount of free E2F exceeds the threshold, E2F activates not only growth-related targets but also pro-apoptotic targets. However, molecular mechanism of how different amount of free E2F differentially regulates target genes is not yet elucidated. The 'quantitative' difference of free E2F seems not sufficient to explain the four differences between deregulated E2F and physiologically activated E2F. The above-mentioned differences strongly suggest the presence of qualitative difference between physiologically activated E2F by 'temporal release' from RB and deregulated E2F induced by 'dysfunction' of the RB pathway [75-77] (Figure 9). The 'qualitative' difference between both of the E2Fs seems to be a useful cue to elucidate how cells discriminate oncogenic growth stimulation from physiological growth stimulation.

5. Conclusion and further research

Accumulating evidence indicates that E2F plays essential roles in cell-fate determination, to grow, stop or die. Together with G1/S gatekeeper RB, E2F governs control of the restriction point, deciding whether to grow or not. Upon normal growth stimulation, physiologically activated E2F facilitates cell proliferation. Upon dysfunction of the RB pathway, deregulated E2F suppresses cell growth by inducing p27^{Kip1} to restrain cells from aberrant cell growth. p21^{Cip1}, induced by p53 through activation of the *ARF* gene, may also contribute to suppression of cell growth. When the arrest mechanism failed to stop the aberrant cell cycle progression, E2F induces apoptosis through activation of p53 and TAp73 to protect cells from tumorigenesis. E2F seems to sense and discriminate between normal growth signals and abnormal growth signals originating from various oncogenic changes, asking cells whether to grow, stop or die.

Deregulated E2F activity specifically exists in cancer cell lines but not in normal growing fibroblasts, suggesting that deregulated E2F activity may be a useful means to discriminate abnormally growing cancer cells from physiologically growing normal cells. Since the generation of deregulated E2F activity is expected to be based on the mechanism of oncogenesis, deregulated E2F activity could be a universal marker to discriminate cancer cells from

normal growing cells. Analyses of atypical E2F targets suggest that deregulated E2F might be qualitatively different from physiologically activated E2F. One of the most intriguing issues in the future studies would be the molecular nature of deregulated E2F. By elucidating the molecular nature of deregulated E2F, we might be able to specifically approach cancer cells without affecting normal growing cells. For this purpose, qualitative difference between deregulated E2F and physiological E2F is eager to be elucidated.

Acknowledgements

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MicroRNAs and lncRNAs as Tumour Suppressors

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Additional information is available at the end of the chapter

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1. Introduction

Cancer is one of the most serious diseases around the world and it is the third leading cause of death, exceeded only by heart and infectious diseases [1]. There are five major steps for cancer development: initiation, promotion, malignant conversion, progression, and metastasis [2]. Cancer is result of process, where somatic cells mutate and escape the controlled balance of gene expression and cellular networks that maintain cellular homeostasis, which normally prevent unwanted expansion. Perturbations in these pathways results in cellular transformation, where cancer cells differ from their normal counterparts in many characteristics, as is loss of differentiation, increased invasiveness, and decreased drug sensitivity [3-4]. There are six primary hallmarks of cancer: unlimited cell proliferation, autonomous growth without the need of external signals, resistance to growth inhibitory signals, escape from apoptosis the ability to recruit new vasculature and increased tissue invasion and metastasis [5]. The formation of cancer is therefore fundamentally genetic and epigenetic disease requiring accumulation of genomic alterations to inactivate tumour suppressor and activate proto-oncogenes [6]. These results in combined interaction of both tumour suppressors, that are not able to inhibit tumour development and protect cells against mutation that initiate transformation, and cancer inducers, which promotes cancer development as initiators of cellular transformation. When cells exhibit abnormal growth and loss of apoptosis, it usually results in cancer formation [2].

Genetic studies have revealed the mutational and epigenetic alterations of protein-coding genes that control DNA damage response, growth arrest, cell survival and apoptotic pathways [4]. Until recent years ago, the central dogma of molecular biology was that genetic information is stored in protein-coding genes with RNA as an intermediate between DNA sequence and its encoded protein [7]. Recent studies suggest that advanced stages of cancer are possessing more severe molecular perturbations and that this could be due to function of non-coding RNAs (ncRNAs), which were previously known only to have infrastructural functions (as

ribosomal RNA, transfer RNA, small nuclear and nucleolar RNA). Eukaryotic genomes are extensively transcribed into thousands of long and short ncRNAs, which are group of endogenous RNAs that also function as regulators of gene expression. They are involved in developmental, physiological as well as pathological processes [7,8].

However, in this review, the following characteristics of ncRNA in human cancers will be summarized: (i) the current understanding of the critical role that lncRNAs and miRNAs may play in cancer as tumour suppressors; (ii) outline current knowledge about some specific lncRNA and miRNAs and their target genes in cancer; (iii) highlight their potential as bio-markers for patho-histological subtype classification; and (iv) highlight their potential as biomarkers and as circulating biomarkers and therapeutic targets in cancer.

Since the majority of research regarding regulatory ncRNAs as tumour suppressor was performed on miRNAs and in lesser extend on lncRNAs/lincRNAs, will this review further focused on these two groups of ncRNAs.

2. Brief overview of non-coding RNAs (ncRNAs)

Classification

Of transcribed eukaryotic genomes, only 1-2 % encode for proteins, whereas the vast majority are ncRNAs that are in more or less functional transcripts. The regulatory ncRNAs are important regulators of gene expression in many eukaryotes and are involved in a wide range of functions in eukaryotic biology [8,9].

Based on their function, ncRNAs can be divided into two groups. First is infrastructural group, with ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs). Second is regulatory group, with microRNAs (miRNAs), piwi-interacting RNAs (piRNAs), small-interfering RNAs (siRNAs), long non-coding RNAs (lncRNAs), large intergenic non-coding RNAs (lincRNAs), promoter-associated small RNAs (PARs), repeat-associated short interfering RNAs (rasiRNAs) and enhancer RNAs (eRNAs) [8,9]. Recent findings suggest that some structural ncRNAs (e.g. snoRNAs) not only have infrastructural function but have regulatory as well [8].

Based on length, the regulatory ncRNAs can be divided in two groups: larger than 200 nucleotides (nt) are lncRNA, lincRNA, eRNA, whereas the others are smaller than 200 nt, with exception of PARs that are 16-30 nt long or up to 200 nt. Distinct classes of small RNAs are distinguished by their origins, and these are: snRNAs, snoRNAs, miRNAs, piRNAs, siRNAs, and rasiRNAs [8,9].

miRNAs and snoRNAs share similarities in processing pathways and protein interaction partners, genomic organization and location, as well as levels of conservation. However, similarities in sub-cellular localization have been also observed, since large proportion of human mature miRNAs have been detected in the nucleus as well as a subset of small RNAs derived from snoRNAs have been detected in the cytoplasm [10].

Functional role

The most widely studied and characterized of all the regulatory ncRNAs are miRNAs. The roles of regulatory ncRNAs, other than miRNAs, in the mediating transcriptional regulation, chromatin remodelling, post-transcriptional regulation, and other processes are less well understood. The contexts of gene regulation by ncRNAs in non-human systems provided insights into how these processes could function in human cells. Regulatory ncRNAs are involved in diverse cellular pathways, such as development and stem cell maintenance, response to stress and environmental stimuli, regulating chromatin structure and remodelling, chromosome architecture and genome integrity, transcription (positive or negative impact), and post-transcription processing (splicing, transport) and most commonly mRNA stability (translation, degradation) [8,9]. Some ncRNAs trigger different types of gene silencing that are collectively referred to as RNA silencing or RNA interference [11].

RNA interference (RNAi)

RNAi is RNA-guided regulation of gene expression, historically known by other names, including post-transcriptional gene silencing. It is believed to be an evolutionary conserved mechanism in response to presence of foreign dsRNA in the cell. A key step in this silencing pathway is the processing of dsRNAs into short RNA duplexes of characteristic size and structure. The enzyme Dicer, which initiates the RNAi pathway, cleaves dsRNA to short double-stranded fragments of 20–25 base pairs (bp), named siRNAs. siRNAs usually possess perfect complementarity to the mRNA of target gene, thus causing its degradation. When the dsRNA is exogenous, coming from infection by a virus with RNA genome or laboratory manipulations, the RNA is imported directly into the cytoplasm where it is cleave by the Dicer. On other hand, the initiating dsRNA could be result of endogenously expressed RNA-coding genes from the genome. Some of small regulatory RNAs are processed in a similar way or with components of RNAi pathway [11].

2.1. Brief introduction to miRNAs

Genomic organization

miRNAs are endogenously expressed small (~22 nt), single-stranded ncRNAs. It is predicted that they constitute ~1-5 % of human genes [1,12] and in an update from August 2012, miRBase v19 was released with a list of 2019 unique mature human miRNAs. miRNAs are encoded as a single gene or gene clusters, with some of miRNA clusters being co-regulated and co-transcribed. Intergenic miRNAs are transcribed as an independent transcription unit, as a monocistronic, bicistronic or polycistronic primary transcripts [13]. Up to 60 % of currently known miRNAs are proposed to be from intronic sequences of either protein coding or non-coding transcription units and suggestion has been made that some miRNAs are also encoded in antisense DNA, which is not transcribed to the mRNA. Intronic miRNA are preferentially transcribed in the same orientation as the host gene and are together with their host transcripts co-regulated and co-transcribed from the same promoter. They are processed from introns, as are many snoRNA. Within the genome, there might be more than one copy of particular miRNA [13,14].

Biogenesis

miRNAs expression is determined by intrinsic cellular factors and diverse environmental variables [1]. As for protein-coding genes it is known, that regulation of miRNA transcription and expression depends on transcription factors and epigenetic mechanisms (e.g. p53, Myc, and myogenin). In general, from genes encoding miRNAs is transcription guided by RNA-polymerase II (Pol II). Resulting primary transcript (several hundred bases to several kilobases), named *pri-miRNA*, forms distinctive hairpin-shaped stem-loop secondary structure and contains poly-A tail and a cap, similarly to protein-coding mRNA. *pri-miRNA* is processed in the nucleus by Drosha, an RNase III enzyme. The resulting 70-nt stem-loop structure called *pre-miRNA* with a 5' phosphate and 3' 2-nt overhang is imported into the cytoplasm by a transporter protein, Exportin 5. The double-stranded RNA portion of *pre-miRNA* is bound and cleaved by Dicer, another RNase III enzyme, which produces a miRNA:miRNA* duplex (a transient intermediate in miRNA biogenesis, 20–25 nt). One of the two strands of each fragment is together with proteins argonaute (Ago), incorporated into a complex called the miRNA-containing ribonucleoprotein complex (miRNP). It is believed that the *guide strand* is determined on the basis of the less energetically stable 5' end. The resulting complex base-pair with complementary 3'-UTR mRNA sequences. The other strand, miRNA* is presumably degraded, although there are increasing evidence that either or both strands may be functional [2,9]. The schematic overview of canonical miRNA biosynthesis pathway has been represented elsewhere [15].

Numerous alternative pathways differing from canonical miRNA biogenesis pathway have been described recently and subset of several diverse longer non-coding RNAs can serve as precursors for miRNAs [10,16]. As an example, intronic miRNAs presumably bypass Drosha cleavage, since through *pre-mRNA* splicing/debranching machinery is produced an approx. 60-nt hairpin precursor miRNA (*pre-miRNA*) that enter biogenesis pathway at the step of Exportin 5 [14]. However, some of the post-transcriptional mechanism include miRNA editing, which is mechanism mediated by adenine deaminase of alteration of adenines to inosines, and not yet thoroughly studied regulations of miRNA, such as export step from nucleus or miRNAs turnover rate [17].

miRNAs mechanism

The functional role of miRNA varies, but the primary mechanism of miRNA action in mammals is believed to be base-pairing to 3'-UTR of target mRNA followed by inhibition of mRNA translation (when base pairing between these two molecules is incomplete) or deadenylation and degradation (perfect complementarity of miRNA:mRNA binding) [9]. Especially in animals, the primary mechanism of miRNA action is reducing mRNA translation and each miRNA can inhibit the translation of as many as 200 target genes. In addition, mRNA can be regulated by more than one miRNA. The cooperative action of multiple identical (multiplicity) or different miRNPs (cooperativity) appears to provide the most efficient translational inhibition. Additional mechanism to increase the specificity of miRNAs is combinatorial control of gene expression, which may be also provided by a set of co-ordinately expressed

miRNAs. Proteins or mRNA secondary structures could restrict miRNP accessibility to the UTRs, or may facilitate recognition of the authentic mRNA targets [12,13,18,19].

There is the prospect that some miRNA might specify more than just post-transcriptional repression [9,13]. miRNAs may also target promoter to regulate transcription through epigenetic mechanism. miRNAs have been paradoxically also shown to up-regulate gene expression by enhancing translation under specific conditions [9].

Biological function

Translational repression, as major mechanism of miRNAs, may in normal cell conditions occur in different ways: as switch off the targets, that is for mRNAs that should not be expressed in a particular cell type, the protein production is reduced to inconsequential levels; as fine-tuners of target expression, that is when miRNAs can adjust protein output for customized expression in different cell types; as neutralizers of target expression, that is when miRNAs act as bystanders, where down-regulation by miRNAs is tolerated or reversed by feedback processes [13]. Role of miRNA can be further divided in three paradigms: combinatorial control (defined as cooperativity), cell-to-cell variation, specific (tissue-specific and/or cell-type specific) and housekeeping functions [20].

Despite the large number of identified miRNAs, the scope of their roles in regulating cellular gene expression is not fully understood [11]. It is believed that miRNAs through negative gene regulation influence at least 50 % of genes within the human genome [9]. Expression profiling of many miRNAs in various normal and diseased tissues have demonstrated unique spatial and temporal expression patterns. Many miRNAs are important at distinct stages of development and have been found to regulate a variety of physiological and pathological processes [11]. miRNAs are involved in a numerous biological processes, such as stem cell division and developmental timing, proper organ formation, embryonic pattering and body growth, proliferation and differentiation, apoptosis, epithelial-mesenchymal-transition (EMT), cholesterol metabolism and regulation of insulin secretion, resistance to viral infection and oxidative stress, immune response etc. [2,11]. All these effects may occur by regulating or being regulated by the expression of signalling molecules, such as cytokines, growth factors, transcription factors, pro-apoptotic and anti-apoptotic genes [21]. With all different genes and expression patterns, it is reasonable to propose that every cell type at each developmental stage might have a distinct miRNA expression profile.

Defining miRNA targets and databases

Up to date, over 2000 human miRNAs have been identified and this number is still growing. All annotated miRNAs are collected in miRBase [22]. The first step in miRNA target identification is usually defining reciprocally regulated miRNA-mRNA or miRNA-protein. Since miRNAs target mRNA mainly by incomplete base-pairing, many computational methods have been recently developed for further identifying potential miRNA targets [23]. Most of these methods search for three criteria in predicting miRNA target genes: first, multiple conserved regions of miRNA complementarities within 3'-UTR of target mRNA (evolutionary conservation); second, interaction between seven consecutive nucleotides in the target mRNAs 3'-UTR and the 1-8 nt ("seed sequence") at the 5' miR-

NA end; third, stability of base pairing and predicted binding energy. Further complicating target site prediction in mammals is the fact that not all 3'-UTR sites with perfect complementarities to the miRNA seed nucleotides are functional. Moreover, mRNAs sites with imperfect seed complementarities can themselves be very good miRNA targets [24,25]. Bioinformatics is therefore much noisier and more prone to false positive and false negative predictions. Among many available programs for predicting mRNA targets for specific miRNA, none of these programs can be used as an independently approach for validating the targets, and all predicted targets must be validated *in vitro* and/or *in vivo*. Thus the gold standard for miRNA target identification is the experimental demonstration that a luciferase reporter fused to the 3'-UTR of the predicted target is repressed by over-expression of the miRNA and that this repression is abrogated by point mutation in the target sequences in 3'-UTR [26,27]. Finaly, expression profiling in human disease gives the starting point for target verification/validation and association to disease prognosis and pathogenesis. All identified disease related miRNAs are listed in The human microRNA disease database (HMDD), where you can search for specific miRNA, for tissue expression of annotated miRNAs, and for disease related miRNAs [28].

2.2. Brief introduction to lncRNA

Genomic organization

LncRNAs are those longer than 200 nt, and many of them can also act as primary transcripts for the production of short RNAs [9]. It is estimated that total number of lncRNA transcripts, including new unexplored, is approx. 15000. Thousands of protein-coding genes in humans harbour natural antisense transcripts (approx. 61 % of transcribed regions show antisense transcription) belonging to the lncRNA, and majority of known lncRNAs in some way overlap protein-coding loci. All these data are giving the importance to lncRNA annotation [29].

Classification

LncRNAs can be classified according to their proximity to protein coding genes. There are five categories of lncRNAs: sense, antisense, bidirectional, intronic, intergenic. Just to mention a few of them, lincRNAs, a class of ncRNAs, exhibit a high conservation between different species; they both up- and down- regulate hundreds of gene expression and participate in the establishment of cell type-specific epigenetic states [9]. Further, ncRNAs were found expressed at enhancer regions, suggesting that some enhancer RNA is also transcribed with an average size of 800 nt; these transcripts are termed eRNAs. Studies propose a possible role for eRNAs as transcriptional activators, however, question remains whether such eRNAs are in fact a subset of the activating lncRNAs. Similar to eRNA, a novel diverse class of ncRNAs has been linked to the promoters, called PARs, ranging from 16-36 nt to 200 nt. It is suggested that they participate in the transcriptional regulation [9]. Most lncRNAs are characterized by low expression levels, low level of sequence conservation, by composition of poly-A tail and without poly-A tail as well as by spliced and un-spliced forms. They are believed to have nuclear localization, but can also accumulate in cytoplasm of cells [3].

Function

lncRNAs may act through diverse molecular mechanisms, and play regulatory as well as structural roles in different biological processes [3]. Many of the identified lncRNAs show spatial- and temporal-specific patterns of expression. Almost every step in the life cycle of genes – transcription, mRNAs splicing, RNA decay, and translation – can be influenced by lncRNAs. Generally lncRNAs have been implicated in gene-regulatory roles, such as chromatin dosage-compensation, imprinting, epigenetic regulation, cell cycle control, nuclear and cytoplasmic trafficking, cell differentiation etc. [7]. A number of studies suggest that lncRNAs are key components of the epigenetic regulatory network [4]. Two general modes of lncRNAs regulation seem to be important: interaction with chromatin remodelling complexes that promote silencing of specific genes; and modulation of splicing factors. Chromatin remodelling guided by ncRNAs contributes to the establishment of chromatin structure and to the maintenance of epigenetic memory. Various ncRNAs have been identified as regulators of chromatin structure and gene expression [30]. Additional mechanisms of action are yet to be revealed [3].

Database

The lncRNA database provides sequence, structural, and conservation evidence for multi-species lncRNAs, together with a list of lncRNAs that are experimentally known to interact with coding mRNAs, harbouring other short ncRNAs and other characteristics of specific lncRNA [29].

3. Involvement of ncRNA in cancer

Three major mechanisms are known to give rise to deregulated ncRNAs function, genetic alterations, epigenetic alterations, and in case of miRNAs, an aberrant miRNA biogenesis machinery. Since brief overview of first two mechanisms is described below, will be here mentioned only aberrant machinery of miRNA processing. Proteins involved in miRNA biogenesis (Drosha, Dicer, Ago) are deregulated in several cancers. Co-factors involved in miRNA biogenesis can be mutated causing consequently deregulation of Dicer; Exportin 5, mediating pre-miRNA nuclear export, is often mutated and truncated, leaving pre-miRNAs within nucleus [31,32].

3.1. Mutations, SNPs and epigenetics of ncRNAs

Cancer cells have different genetic and epigenetic changes from their normal counterparts and the role of ncRNAs in mediating these differences is beginning to emerge. Specific genetic polymorphisms are associated with the risk of developing several types of cancer [7-9]. Multiple studies have identified small-scale and large-scale mutations and genomic alterations affecting also noncoding regions of the genome. Some of these mutations are structural alterations, rearrangements and chromosomal translocation, amplification, loss of heterozigocity and copy-number variation, nucleotide expansion, and single-nucleotide polymorphisms (SNPs), and they are linking distinct types of mutations in ncRNA genes with diverse

diseases [7]. First, lncRNA have already been implicated in human diseases such as cancer and neurodegeneration [33]. Second, approx. half of miRNA genes are encoded in genomic region prone to cancer-associated rearrangements or in fragile chromosomal sites (amplified, deleted or rearranged) that are often associated with cancer, such as ovarian and breast carcinomas, and melanomas [8,11]. Third, presence of SNPs in miRNAs, where disruption of miRNA target interaction either in the miRNA gene or its target site (3′-UTR mRNA) can lead to complete gain or loss of the miRNA function or target gene thus causing disease [34,35]. In contrast to the miRNA target sites in mRNA transcripts, where the potential of variation is huge, variants identified in miRNA precursor sequences tend to be rarer [36]. The presence of SNPs in *pri-miRNA* or *pre-miRNA* can in addition affect the processing of miRNAs, their expression and/or binding to target mRNA [27,37]. Forth, recent advances in miRNA research have provided evidence of a miRNA association with epigenetic mechanisms activated in diseased human tissues [38]. Heritable changes in gene expression that do not involve coding sequence modification are referred as epigenetics. Gene regulation by ncRNAs was considered as an epigenetic mechanism, but ncRNAs can be regulated by the same mechanism in which they participate [39]. DNA methylation, one of the two major epigenetic mechanisms, leads to gene silencing, and serves as an alternative mechanism of gene inactivation. The aberrant DNA methylation of gene promoters has been shown to result in the inactivation of tumour suppressor genes [40]. For an example, *miR-34* family is a family of tumour suppressors' genes, with *miR-34a* being deregulated by DNA methylation in both epithelial and haematological cancers. *miR-34* is an important component of the p53 tumour suppressor network, and p53 is a predicted target for members of the *miR-34* family. *miR-34a* reinforces the tumour suppressor function of p53, transactivation of *miR-34a* by p53 was also shown to promote apoptosis [11,41-44]. Another example is that *miR-29s* could target two enzymes of methylation process, DNMT3A and DNMT3B [39]. Antisense ncRNAs have been recently showed to be implicated in the silencing of tumour suppressor genes through epigenetic remodelling events [30]. All these miRNAs abnormalities suggest that they play a broad role in cancer pathogenesis.

3.2. Promising role of ncRNAs in cancer: As cancer-subtype classifiers and detection in body fluids

ncRNAs have been recognized as gene-specific regulators. They are similar in activity to a large number of protein transcription factors that are known to be critical in the transformation of cells to a malignant state. Majority of research has been involved in defining the role of miRNAs in cancer; however, lincRNAs have been shown to play role in tumour development by promoting the expression of genes involved in metastasis and angiogenesis [9]. Genome-wide analyses have shown that ncRNAs have distinct signatures specific for a certain cancer type. Importance of combining ncRNAs with other biomarkers for cancer detection and prognosis would improve cancer risk assessment, detection, and prognosis. Thus, there is a need to combine genomic mutations with ncRNA markers to develop marker panels for more accurate risk assessment and early diagnosis [7-9].

Most cancers are diagnosed in advance stages, leading to poor outcome. Intense investigation is going on seeking specific molecular changes that are able to identify patients with early cancer or precursor lesions [1]. Genome-wide expression profiling has examined miR-

NAs in preneoplasia or their usefulness to predict progression from preneoplasia to cancer. Several lines of evidence suggest the potential usefulness of ncRNAs, particularly miRNAs: first, as signature of early events in carcinogenesis and as biomarkers in early cancer detection, second, as differential indicators of benign tumours, preneoplasia, and neoplasia, and third, that miRNAs and perhaps other ncRNAs might be useful in determining which preneoplastic lesions are likely to progress to cancer. Distinguishing benign diseases and certain non-precancerous lesions from precancerous lesions and metastatic tumours would improve patient outcomes, survival, and reduce patient discomfort [45]. Characterization of ncRNAs involved in the development or maintenance of oncogenic states may therefore define ncRNAs as early biomarkers for the emergence of cancer, and could have have an impact on the development of tools for disease diagnosis and treatment [30].

miRNAs are believed to be promising potential biomarkers for cancer diagnosis, prognosis and targets for therapy. As potential markers for diagnosis are better classification factors than mRNAs. miRNAs seems to be evolutionarily selected gene regulatory molecules, their expression profiles might therefore be rich in gene regulatory information. Only small percentage of the 16000 genes on the mRNA-expression arrays are regulatory molecules. This difference may be responsible for more efficient microRNA expression arrays in classifying cancer than mRNA-expression arrays [21,45,46]. Some of the key features of miRNAs that make them useful as potential biomarkers can be briefly summarized. First, expression patterns of miRNAs in human cancers appear to be tissue specific. Second, miRNA profiles appear to reflect developmental lineage and differentiation state of the tumours. Third, miRNAs can successfully classify poorly differentiated tumours with high accuracy (~70 %). In contrast, mRNA profiles in the same set of specimens had an accuracy of only 6 %. Therefore, a combination of both miRNA and mRNA profiling data has the potential of enhancing accuracy of tumour classification. Forth, miRNAs can also be profiled and quantitatively measured in formalin-fixed paraffin-embedded tissues. And last, miRNAs are stable in human body fluids of plasma and serum and can be quantitatively measured in microliter quantities of human sera or plasma using qPCR. [45-47].

Highly stable cell-free circulating nucleic acid (cfCNA), both RNA and DNA, has been discovered in the blood, plasma, and urine in humans. Since there is good correlation between tumours and genetic, epigenetic and/or transcriptomic changes and alterations in cfCNA levels, it gives a usefulness of cfCNA as biomarkers for clinical applications. Release of cfCNA in body fluids is probably related to apoptosis and necrosis. Circulating RNAs are stable in serum and plasma in spite of high amounts of RNAase in blood of cancer patients [48]. They are packed in microparticles, of which the most analyzed are in recent years exosomes [3,49,50]. Tumour derived exosomes are small membrane vesicles of endocytic origin released by the tumour and found in peripheral circulation. Several recent reports showed that exosomes could be an important resource of cf-lncRNA/cf-miRNA in serum or plasma [51]. Small size, relative stability and resistance to RNAase degradation make the miRNAs more superior molecular markers than mRNAs [1]. Using non-invasive diagnostic procedures, the extraction and reliable determination of cf-miRNAs, circulating in body fluids like plasma, serum, and others, could serve as circulating tumour biomarkers [52,53].

LncRNAs show greater tissue specificity compared to protein-coding mRNAs, making them attractive in the search of novel diagnostics and/or prognostics cancer biomarkers in body fluid samples. For an example, lncRNA PCA3 was initially identified as over-expressed in prostate tumours relative to benign prostate hyperplasia and normal epithelium. It was latter showed that is very specific prostate cancer gene, whose mechanism is not yet identified, but it can be detected in urine samples and has been shown to improve diagnosis of prostate cancer [3].

3.3. ncRNAs can act as both tumour suppressor genes and oncogenes

There are different ways in which miRNAs appear to be involved in cancer: as tumour suppressors, as oncogenes, or as agents involved in affecting genome stability. Below is discussed role of miRNAs acting both, as tumours suppressor and as oncogenes, since are much more investigated in this field than are lncRNAs. Care must be taken in assigning oncogenic or tumour suppressor activity to a miRNA, since miRNA expression patterns are highly specific for cell-type and cellular differentiation status. The same miRNA can function as tumour suppressor in one cell type and as potential oncogene in other cell type. Some of the aberrant miRNA expression observed in tumours may also be a secondary consequence of the loss of normal cellular function that accompanies malignant transformation. Up- or down-regulation of a miRNA in a given tumour type is not obvious a causative role in tumorigenesis [6].

The increased expression of oncogenic miRNAs appears to act in a manner analogous to an oncogene. Over-expression of oncogenic miRNAs are presumed to function by down-regulating the levels of protein product of target tumour suppressor gene or by reduction of tumour suppressor processes, such as apoptosis [2,6,20]. A loss of expression of tumour suppressor miRNA may lead to elevated levels of the protein products of target oncogenes [6], activation of an oncogenic processes, such as proliferation [2,20]. MicroRNAs with anti-proliferative and pro-apoptotic activity are likely to function as tumour suppressors and thus may be under-expressed in cancer cells. Figure 1 represents schematic overview of miRNAs acting as tumour suppressors or oncogenes in comparison to non-cancerous cells.

There should be at least four type of evidence before assigning tumour suppressor function to ncRNAs: (i) data about widespread deregulation in diverse cancer, (ii) gain or loss of function in tumours owing to deletion, amplification or mutation, (iii) direct documentation of tumour suppressing activity using cell line or animal models, (iv) the identification and verification of cancer relevant targets that define mechanisms through which miRNAs participate in onco-genesis [6].

4. ncRNAs as potential therapeutic targets in cancer

4.1. RNAi in therapeutic applications

Using RNAi approaches, ncRNAs may in future serve as therapeutic targets. For ncRNA that is under-expressed and possess tumour suppressor function, re-introduction of the mature
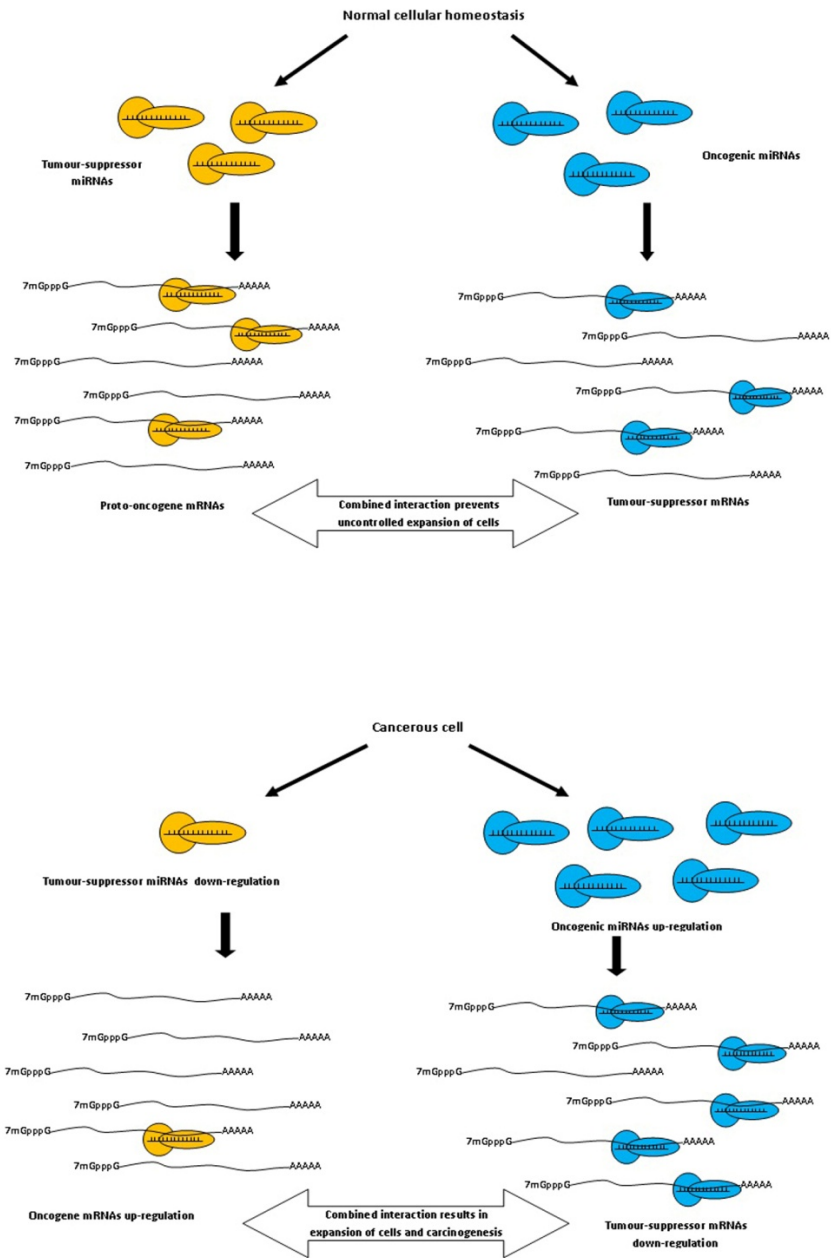
Figure 1. Schematic overview of miRNAs acting as tumour suppressors or oncogenes.

ncRNA into the affected tissue would restore the regulation of the target gene. By contrast, over-expressed ncRNA with oncogenic function could be down-regulated by reducing mature ncRNA level by its direct targeting [54].

Due to the interferon response it is difficult to introduce long dsRNAs into mammalian cells, however, the use of RNAi as a therapeutic approach has been successfully used. Among the first applications to reach clinical trials were in the treatment of macular degeneration and respiratory syncytical virus infection, reversal of induced liver failure in mouse models, antiviral therapies, neurodegenerative diseases, and cancer. Cancer was treated by silencing up-regulated genes in tumour cells or genes involved in cell division. A key area of research in the use of RNAi for clinical applications is the development of a safe delivery method, which to date has involved mainly viral (lentivirus, adenovirus, adeno-associated virus) and non-viral (nanoparticles, aptamers, stable nucleic-acid-lipid particle, e.g.) vector systems similar to those suggested for gene therapy [55].

4.2. ncRNAs with tumour suppressor function as therapeutic targets

Replenishing small RNAs/miRNAs

Pharmacological manipulation of miRNAs is still in its infancy; however, the correlation between the expression of miRNAs and their effects on target oncogenes, on tumorigenesis, and on the proliferation of cancer cells has gained experimental support. miRNAs are small molecules, making their *in vivo* delivery feasible. It has been shown that miRNAs can be delivered systematically, and can reduce invasion, proliferation and growth as well as induce radio-sensitivity and resistance. miRNAs may therefore serve as therapeutic targets in the future.

For miRNA that is under-expressed, re-introduction of the mature miRNA into the affected tissue would restore regulation of the target gene. For this purpose, artificial miRNA (miRNA-mimic) have been developed to enhance the expression of beneficial miRNAs or the introduction of short hairpin duplex, similar to *pre-miRNA*, into the cell. This suggests that individual miRNAs are potential therapeutic agents, provided that their expression or delivery can be targeted to appropriate tissue. Most of the developed protocols have used local administration in easily accessible tissue; systemic delivery has also give some promising results; the major challenge remains tissue and cell-type specific targeting [56].

miRNA mimic can only last a couple of days and the long term biological effects were not observed very effectively. To overcome this, the cells were infected with a lentivirus that expressed mature miRNAs. This generated stable cell expressing miRNAs. miRNA mimics and lentiviral miRNAs showed great potential in restoring tumour suppressor miRNAs. However, viral and non-viral delivery systems have been developed. Viral vector-directed methods show high gene transfer efficiency, but have some limitations. However, non-viral gene transfer vectors have been also developed: cationic liposome mediated gene transfer system, lipoplexes, neutral lipid emulsion, etc. [57].

Expression of miRNA-mimic would simultaneously suppress many gene targets. miR-NAs-mimic would be useful in conjunction with standard chemotherapy or radiotherapy,

by influencing drug resistance or enhancing responsiveness to therapy. Current limitation is need for improvement of efficiency of delivery to target tissue, for systemic drug administration, potential inhibition of non-target genes ("off-target effect"), redundancy among miRNAs efficacy, potential toxicity and immunogeneic responses. However, studies introducing miRNAs strategies to inhibit cancer propagation in animal models are showing promising results [32].

Examples for miRNA

Therapeutic delivery to animal models was demonstrated using miRNA-mimics of the tumour suppressor miRNAs, *miR-34a* and *let-7a*, both of which are often down-regulated or lost in lung cancer. It has been shown that re-introduction of *let-7* directly represses cancer growth in the lung [58] and that development of chemically synthesized therapeutic *miR-34a* and lipid-based delivery vehicle block tumour growth in mouse models of non-small-cell lung cancer (NSCLC) [59]. Systemic treatment of these mice led to significant decrease in tumour burden. Mice treated with *miR-34a* displayed a 60 % reduction in tumour area compared to mice treated with a miRNA control. Similar results were obtained with the *let-7* mimic [60].

Targeting lncRNAs

Successful inhibition of lncRNAs seems to be more difficult than inhibition of miRNAs. Our growing knowledge of other ncRNAs might exploit in future to develop new therapeutic strategies not only against cancer, but also for other diseased states. The findings regarding lncRNA and Alzheimer disease are attracting the attention of pharmaceutical and biotechnology industries [8]. Therapy using small RNAs that targets ncRNA transcripts, such as eRNAs or PARs, may represent a new way to treat disease conditions caused by epigenetic changes [9].

Targeting both, lncRNAs and miRNAs

Another possible approach for manipulation of ncRNAs level may also be by altering DNA methylation. As mentioned above, DNA methylation is a crucial mechanism associated with epigenetic regulation. It has been shown that in cancer cells treated with DNA demethylating agent reactivation of certain miRNAs occurs [40]. ncRNAs mediated therapy may also be useful in combination with DNA methyltransferase inhibitors that are other way toxic [39].

5. ncRNAs as tumour suppressor in different types of cancers

5.1. miRNAs as tumour suppressors

In the following section, down-regulated miRNAs will be describe and miRNAs with suggested tumours suppressive roles in different types of cancer. However, down-regulation does not ncessary mean that miRNA is tumours uppressor.

Hematological cancers

Leukaemia. Chronic lymphocytic leukaemia (CLL) is characterized by overexpression of the protein Bcl-2 in B cells and represents the most common human leukaemia. In less than 5 %

of cases, over-expression of Bcl-2 is due to a translocation of the Bcl-2 gene, whereas for the majority of CLL cases no explanation for the deregulation of Bcl-2 has been reported. It has been demonstrated that mutations in genomic regions containing miRNAs were associated with disease progression in a number of CLL patients. In this type of cancer, *miR-15* and *miR-16* expression is often reduced and indeed, one of the first associations between miRNAs and cancer development was observed for *miR-15* and *miR-16* in CLL [46]. Both miRNAs are located in a 30 kb region on chromosome 13 that had been found deleted in more than half of B cell CLL (chromosome 13q14 deletion) [8,46], and *miR-15a* and *miR-16-1* have been shown to be deleted or translocated in approx. 65 % of CLL patients [2,11]. Several papers indicate that miRNA regulates cell growth and apoptosis. Indeed, over-expression of *miR-15* and *miR-16* directly inhibit anti-apoptotic Bcl-2, a key player in many types of human cancers, and thus activate apoptotic processes [2,11]. However, it was further demonstrated that other mutations in miRNA genes are frequent in CLL; many mutations were located in the flanking sequence of *pre-miRNA*, thus cell culture assay indicated that a point mutation of the *miR-16-1* precursor abolishes expression of mature *miR-16* [21]. Few other miRNAs were also recognized as tumour suppressors in CLL. *miR-29a* and *miR-29b* are associated with fragile site FRA7H that is not associated with any known tumour suppressor gene. Over-expression of *miR-29b* may target TCL1 and reduces anti-apoptotic Mcl-1 protein in CLL patients [11]. Another well-known tumour suppressor was analysed. Low expression of *miR-34a* in CLL was found to be associated with p53 inactivation, impaired DNA damage response, apoptosis resistance and chemotherapy-refractory disease irrespective to p53 mutation (cases with CLL with p53 mutation are resistant to chemotherapy). It was latter showed that *miR-34a* is induced by p53. In another type of leukaemia, particularly acute myeloid leukaemia, an inverse correlation between *miR-34b* and CREB expression has been observed. After restoring expression of *miR-34b*, cell cycle abnormalities, reduces growth and altered CREB expression has been observed, suggesting tumour suppressor potential of this miRNA [47].

Lymphoma.miR-142 gene was found at the junction of the t(8;17) translocation, which may contribute to the progression of an indolent lymphoma into aggressive B-cell leukaemia [21].

Breast cancer

Breast cancer is one of the most important cancers in adult females. *miR-125b, miR-145, miR-21* and *miR-155* were significantly reduced in breast cancer tissue and this expression was correlated with specific breast cancer pathologic features, such as tumour stage, proliferation, oestrogen and progesterone receptor expression, and vascular invasion. Some of these miRNAs act as oncogenes (e.g. *miR-21*) in many cancer types, so it is suggested that some miRNAs act as tumour suppressors in one cancer type and as oncogenes in another [2]. *miR-125b-1* is located on a fragile site on chromosome 11q24, which is deleted in a subset of patients with breast cancer [61]. Down-regulation of *mir-221* in breast cancers was detected, whereas germ line mutation in mature *miR-125a* is highly associated with breast cancer tumorigenesis, suggesting its tumour suppressor role. *miR-125a* is also down-regulated in human breast cancer and when over-expressed post-trancriptionally regulates CYP24 resulting in an anti-proliferative effect. Ectopic expression of *miR-30e* suppresses cell growth in breast cancer, probably through targeting Ubc9 [47]. *miR-17-5p* was down-regulated in breast

cancer cells, and enhanced expression decreased tumour cell proliferation [11]. Cyclin D1 has been identified as a direct target for *miR-17/20* that functions to suppress proliferation of breast cancer cells. Additional miRNAs have also been shown to be down-regulated and have tumour suppressor function in other types of cancer: *let-7, miR-145, miR-34a, miR-214,* and *miR-205.* MicroRNAs, *miR-31, miR-126, miR-146a/b, miR-206* and *miR-335* have been shown as anti-metastatic miRNAs [62,63].

Colorectal Cancer (CRC)

Colorectal cancer is the third most commonly diagnosed cancer in the world, but it is more common in developed countries. Also in colorectal neoplasia miRNAs expression is associated to the tumour formation. Reduced expression of *miR-143* and *miR-145* have been shown to be a frequent feature of colorectal tumours (adenomatous and cancer stage) when compared to normal mucosa [2,6,64]. A tumour suppressive role of *miR-143* has been elucidated in the epigenetic aberration of CRC with DNMT3A as a target. Restoration of *miR-143* expression in CRC decreases tumour cell growth and down-regulates DNMT3A expression [47]. *let-7* has been implicated in development of colon cancers and progression of colorectal cancers; together with *miR-143* and *miR-18a* was observed to be down-regulated and target KRAS [11, 64]. *miR-145* has been proposed as a tumour suppressor and it has been shown that target IRS-1, and when over-expressed it dramatically inhibits the growth of colon cancer cells. A ubiquitous loss of *miR-126* expression in colon cancer lines was observed and its reconstitution resulted in a significant growth reduction. Also, in a panel of matched normal colon and primary colon tumours, each of the tumours demonstrated *miR-126* down-regulation [64]. Down-regulation of *miR-200* family is a hallmark of EMT as well as up-regulation of ZEB1 transcription factor, and it was shown that in colorectal cells ZEB1 directly suppress tran-scription of *miR-141* and *miR-200c* [57]. It was found that *miR-192* and *miR-215* was down-regulated in CRC, and their anti-proliferative effect was identified in CRC cell lines. It was further defined that both are regulated by p53 and that their targets are a number of transcripts that regulate cell cycle checkpoints [57]. An inverse correlation between COX-2 and *miR-101* was reported in CRC cell lines, and this was further confirmed in colon cancer tissue and liver metastases derived from CRC patients. *miR-16, miR-125b, miR-31, miR-133b,* and *miR-96* were along with already mentioned miRNAs showed to be down-regulated in colorectal cancer [1].

Aberrant DNA methylation may further induce silencing of specific miRNAs in CRC. While methylation of *miR-129* and *miR-137* CpG islands is frequently observed in CRC, is methylation of *miR-9-1* associated with the presence of lymph node metastasis and expression of *miR-9* in CRC inversely correlated with the methylation of its promoter regions [47]. In human colon cancer cell lines, *miR-34a* was showed to participate in the apoptotic program triggered by p53 activation and loss of *miR-34a* expression occurs frequently in cancer cells. p53 directly binds to the genomic region defined as the *miR-34a* promoter with consequent *miR-34a* targeting genes of cell cycle, DNA repair, mitotic checkpoint, DNA integrity checkpoint, cell prolifera-tion, and angiogenesis. Among the down-regulated targets of *miR-34* family were well-characterized p53 targets, such as CDK4/6, cyclin E2, E2F5, BIRC3 and Bcl-2. These effects were nearly identical irrespective of whether *miR-34a, miR-34b* or *miR-34c* was introduced into cell lines. Another target was identified for *miR-34a*, SIRT1, negative regulator of apoptosis.

miR-34a promoter hyper-methylation was observed in 3 of 23 cases of colon cancer, *miR-34b/c* were found to be epigenetically silenced in 9 of 9 cell lines and in 101 of 111 primary CRC tumours [42,64].

Gastric cancer

Gastric cancer is the fourth most common cancer and the second leading cause of cancer death in the world. It was reported that loss of Ago2, which leads to premature stopping of miRNAs biogenesis and general deregulation of miRNAs expression, was observed in 40 % of human gastric cancer patients with high microsatellite instability. A number of miRNAs were reported to be down-regulated. Among these, *miR-141* was significantly low expressed in 80 % of primary gastric carcinoma compared to non-cancer adjacent tissue. It targets FGFR2 and its down-regulation means proliferative potential and poor differentiation of gastric cancer cells [65]. It appears that down-regulation of *miR-451* is related to the worse prognosis of the gastric cancer patients. Over-expression of *miR-451* in gastric cancer cells regulates the oncogene MIF production, reduces cell proliferation and increases sensitivity to radiotherapy [47]. *miR-101* was down-regulated in gastric cancer cells, its targets are: EZH2, Cox-2, Mcl-1, and Fos [65]. Other potential tumour suppressor miRNAs in gastric cancer are: *miR-181b/c* and *miR-432AS*, and for *miR-181* it was proposed that modulate expression of Bcl-2. Low level or loss of expression in gastric cancer also showed *let-7a, miR-486*, and *miR-449*. However, a proposed role for *miR-107* and *miR-126* is controversial in gastric cancer, either tumour-suppressive or oncogenic [66].

An epigenetic silencing of *miR-512-5p* was observed in gastric cancer cells. As its target is was shown anti-apoptotic protein Mcl-1 and after epigenetic treatment (demethylation), it results in apoptosis of gastric cancer cells [65].

The association between genetic polymorphism of *miR-196a-2* and risk of gastric cancer has been identified; it was found that the variant homozygous genotype of *miR-196a-2* was associated with significantly increased risk of gastric cancer [65].

Pancreatic cancer

Pancreatic cancer is the eighth most common cause of cancer-related deaths worldwide and it has a poor prognosis for all stages. It is usually diagnosed at advent stages, therefore it is an urgent need to find some specific biomarkers and key components of carcinogenesis. Several miRNAs were reported to suppress metastasis. In pancreatic cancer cell lines, *miR-146a* was decreased compared to normal ductal epithelial cell line, and its ectopic expression inhibited invasive capacity of pancreatic cancer cell lines. *miR-96* is believed to be a potential tumour suppressor through targeting KRAS. It is significantly down-regulated in pancreatic cancer, its ectopic expression induces apoptosis, inhibits cell proliferation, migration and invasion. In human clinical samples there is observed inverse correlation between *miR-96* and KRAS. Further, ectopic expression of *miR-520h* has inhibitory effect on pancreatic cancer cell migration and invasion, *miR-20a*, with metastasis-suppressing effect, is reduced in pancreatic cancer and its cell lines. [67]. In pancreatic ductal carcinoma, *miR-345, miR-139*, and *miR-142-p* were the most down-regulated miRNAs in tumour tissue compared to normal tissue [1]. Other potential

tumour suppressor miRNAs involved in pancreatic cancer are: *miR-100, miR-181a*, and *miR-15b*, but are as well as *miR-200* family up-regulated [67].

miR-200 family, potential tumour suppressors, is up-regulated in pancreatic cancer. Low expression of *miR-200* family genes and higher expression of their target is common in different cancers. However, most pancreatic cancer cell investigations showed hypo-methylation of *miR-200a/b* and its over-expression, in contrary its targets are hyper-methylated, suggesting that this pathway is not involved in metastases in most pancreatic cancer [67]

Hepatocellular Carcinoma (HCC)

Primary liver cancer mainly refers to HCC, which is one of the most common malignant tumours in liver and accounts for 85-90 % of primary liver cancers. Cyclins D2 and E2 were validated as direct targets for *miR-26a*, which exhibit reduced expression in HCC [68]. In another early study on animal models, down-regulation of number of miRNAs has been detected, including known tumour suppressor miRNAs, such as: *miR-15/16*, *miR-34a*, *miR-150* and *miR-195* [69]. *miR-122*, which represents 70 % of all liver miRNAs, was found to be frequently down-regulated in HCCs. Loss of *miR-122* expression in tumour cells segregates with specific gene expression profiles linking to HCC progression. *miR-122* is specifically repressed in a subset of primary HCCs that are characterized by poor prognosis and is therefore suggested as tumour suppressor miRNA [47,68]. As one of *miR-122* targets, cyclin G1 was identified, through its regulation *miR-122* influences p53 protein stability and transcriptional activity. Two other *miR-122* targets, which promote tumorigenesis, are SRF and IGF1R. The cellular mRNAs and protein levels of Bcl-w were also repressed by *miR-122*. Other pro-apoptotic functions were assigned for *let-7* through targeting Bcl-xL, for *miR-101* through targeting Mcl-1, and for *miR-29* through targeting Mcl-1 and Bcl2. *miR-195* was significantly reduced in HCC tissues and cell lines, it suppress tumorigenicity through targeting cyclin D1, CDK6, and E2F3. CDK6 was showed to be also target for *miR-124*, which is silenced through CpG methylation in HCC; *miR-124* in addition targets vimentin, SET, and MYND. *Let-7g* inhibits the proliferation of HCC by down-regulating c-Myc. Methylation of *miR-1* in HCC results in enhanced tumour cell growth, probably through release of its oncogenic targets c-Met, FoxP1, HDAC4. *miR-223* targets Stahmin 1 and is down-regulated in HCC whereas *miR-375* inhibits the proliferation and invasion of HCC cells by targeting Hippo-signalling effectors YAP [68,70]. Research on expression profiling in HCC and adjunct non-tumour tissue defined that *miR-199a*, miR-195, miR-199a, miR-200a*, and *miR-125a* were also under-expressed in HCC tissue [2]. Anti-metastatic functions were showed for *miR-122* by targeting desintegrin and metalloprotease, and *let-7g* by targeting type I collagen A2. c-Met is target for *miR-1, miR-34a, miR-23b* and *miR-199a-3p*, and all of these miRNAs are down-regulated in HCC [68,70].

Lung cancer

Lung cancer is one of the most common cancers of adults and is also leading cause of cancer-related deaths in many economically developed countries [2]. *Let-7* family is a family of miRNAs, whose genes map to different chromosome regions that are frequently deleted in lung cancer [71]. Significantly worse survival was observed among patients with low expres-

sion of *let-7a-2* compared to those with opposite expression pattern, independent of disease stage [2]. Over-expression of *let-7* in lung adenocarcinoma cell lines inhibited cancer cell growth and reduced cell cycle progression. These findings reflect that *let-7* mediates tumour suppressive function [2,45]. It has been shown that *let-7* regulates the expression of several oncogenes, RAS, MYC, HMGA2, and cell-cycle progression regulators, CDC25, CDK6, cyclin D2. *let-7* is down regulated in lung tumours and its expression anti-correlated with that of RAS relative to the normal lung tissue [46,71]. In animal models, ectopic *let-7g* expression reduces tumour burden and intranasal administration repress lung adenocarcinoma. A SNP in *let-7* complementary site 6 in 3'-UTR of its target KRAS is significantly associated with increased risk for NSCLC among moderate smokers [71]. The *let-7* family was subsequently found to be deregulated in a large number of tumour types [46].

Mutations in EGFR gene are more frequent in NSCLC patients who never smoked tobacco; a significant down-regulation of *miR-145* has been demonstrated in the cancer tissues of these patients. Restoration of this miRNA can inhibit cancer cell growth in EGFR mutant lung AD [47,71]. *miR-128b* has been also showed as direct regulator of EGFR, whereas *miR-128b* loss-of-heterozigocity is frequently found in NSCLC. *miR-7* is frequently down-regulated in lung cancer, it suppress EGFR and Raf1, it attenuates activation of Akt and ERK, suggesting that is negative regulator of EGFR pathway [71].

It was proposed that *miR-140* regulates PDGF in lung cancer development, but this has not yet been thoroughly investigated. *miR-29* is down-regulated in lung cancers, its targets are Mcl-1, DNMT3A and DNMT3B, suggesting that it is pro-apoptotic and that it has role in regulating epigenetic DNA methylation. Further, in lung cancer cells induction of *miR-34* results in apoptosis; miRNA profiling revealed that *miR-34a/b/c* are directly correlated with expression of p53. Decreased expression of *miR-126* and increased expression of VEGFA was found in various lung cancer cell lines. Introduction of *miR-126* down-regulates VEGFA, inhibits growth, and reduces average tumour weight. Mouse model of lung adenocarcinoma showed that *miR-200* family possess anti-metastatic abilities [5]. *miR-125b-1* is located on a fragile site on chromosome 11q24 which is deleted in a subset of patients with lung cancer [61].

Human brain cancer

The phrase "brain tumours" describes an inhomogeneous collection of various tumours of the brain, which represents primary tumours of nervous central system or metastases. Glioblastomas (belongs to family of gliomas) are the most frequent occurrence and malignant form of primary brain tumors in contrary to medulloblastomas, which have a better prognosis [2,11]. Several articles have described the effects of ectopic miRNA modulation on medulloblastoma cell proliferation and growth.

Rescued expression of *miR-9* and *miR-125a* were shown to promote medulloblastoma cell growth arrest and apoptosis by targeting TrkC, whereas *miR-29* has been shown to be down-regulated in neuroblastoma and brain tumour [47,72]. Further, *miR-34a* induces apoptosis in neuroblastoma cells, possibly by targeting the transcription factor E2F3 [11]. Transient transfection in medullablastoma cells with *miR-34a* strongly inhibited cell proliferation, cell cycle progression, cell survival and cell invasion. *miR-34a* was shown to inhibit c-Met, Notch-1,

Notch-2 and CDK6. Ectopic up-regulation of *miR-124* was shown to inhibit cell proliferation, and it was demonstrated that *miR-124* target and regulates CDK6. *miR-125b*, *miR-324-5p*, and *miR-326* over-expression inhibit medulloblastoma cell growth by targeting Hedgehog signalling pathway. *miR-128* also inhibits growth by targeting Bmi-1 oncogene. Another miRNA, *miR-199b-5p* negatively regulates proliferation and cell growth [72].

In contrary to medulloblastomas, are gliomas the most common and deadly primary human brain tumours, and its subtype glioblastomas are highly invasive, very aggressive, and one of the most incurable [2]. *miR-181a, miR-181b* and *miR-181c* were originally identified as down-regulated in glioblastoma cells and tumours when compared to normal brain controls. *miR-181a* and to a greater extent *miR-181b* were subsequently described as tumour suppressors that inhibit growth and induce apoptosis of glioma cells. *miR-181a* over-expression down-regulates Bcl-2. Several other miRNAs have been implicated in glioma malignancy as tumour suppressors. *miR-15b* was suggested to target CCNE1, the gene encoding cyclin E1, however, a direct link between CCNE1 down-regulation by *miR-15* and cell cycle regulation was not demonstrated. *miR-146b* was shown to inhibit glioma cell migration and invasion, and was identified as one of miRNAs that is significantly deregulated in human glioblastoma tissue. *miR-146b* over-expression or knock-down did not affect the growth of human glioblastoma cell line, while it significantly reduced the migration and invasion of one glioblastoma cell line. MMP16 was identified as one of the downstream targets of *miR-146b*. *miR-125b* was shown to induce cell cycle arrest and inhibits CDK6 and CDC25A expression in glioma cell lines. However, another study suggested oncogene function for *miR-125b*. *miR-153* decrease cell proliferation and increased apoptosis (pro-apoptotic miRNA), it inhibited Bcl-2 and Mcl-1. *miR-17* and *miR-184* were identified as two miRNAs with reduced expression in higher grades of glioblastomas. Their over-expression inhibited viability, proliferation, invasion and decreased expression of AKT 2 and several other genes. *let-7* over-expression effect was investigated in glioma cells, its transfection reduced expression of RAS oncogenes, proliferation in vitro, migration of the cells, and reduced the size of tumours generated [73].

Head and Neck Squamous Cell Carcinoma (HNSCC)

Head and neck tumours are a heterogenous group with different behaviour at the various sites arising from anatomical factors, cell-type variation, and differences in exposure to risk factors including tobacco, alchocol, and viruses [74]. Head and neck squamous cell carcinoma is represented by epithelial cancers of the oral cavity, pharynx, nasal cavity, paranasal sinuses, salivary glands and larynx [75]. Studies were made in expression profiling regarding different sites of head and neck tumours, tongue, tonsil, larynx, hypopharynx, nasopharynx, saliva, oral cavity, salivary gland and animal models. Several miRNAs were identified as down-regulated, and for some of their target genes were validated [74].

Low expression of *miR-205* is significantly associated with local-regional recurrence independent of disease severity at diagnosis and treatment. Combined low expression of *let-7d* and *miR-205* is significantly associated with poor survival. In nasopharengyal carcinoma down-regulation of *miR-34* family, *miR-145* and *miR-143* was also observed [47]. Tumour suppressive role in HNSCC has been suggested for *let-7* family, *miR-125a/b*, *miR-200*, *miR-133a/b*, and *miR-100* [75]. General down-regulation of *miR-1*, *miR-133a* and *let-7b* was also observed [74].

Reduced expression for majority of members of the *let-7* family (except *let-7i*) was observed in HNSCC. KRAS and HMGA2 have been characterized as targets for *let-7* [75]. Notable among down-regulated was also *miR-98*. However, another group identified *miR-98* as another regulator of an oncogene HMGA2 [74]. A possible molecular mechanism of *miR-125a/b* down-regulation might be through targeting ERBB2, since its higher level of expression was observed in oral SCC. Other target were also suggested for *miR-125*, namely KLF13, CXCL11 and FOXA1 [74,75]. Down-regulation of *miR-133a/b* in primary HNSCC may further contribute to increased cell proliferation and decreased apoptosis. PKM2 has been validated as cellular target for both, *miR-133a* and *miR-133b*, and increased expression of PKM2 has been associated with cancer progression. Finally, *miR-100* has been observed at suppressed levels in primary HNSCC and derived cell lines. A few of its targets are known, namely FGFR1, MMP13, ID1, FGFR3, EGR2. The exact role has to be investigated yet, but suggestion has been made that down-regulation of *miR-100* means higher rate of cell proliferations.

Deregulation of miRNAs in cancer can occur through epigenetic changes (promoter CpG island hyper-methylation in the case of *miR-200* family) [8]. Suppressed *miR-200a* was detected in primary oral SCC. Members of *miR-200* family inhibit EMT by directly targeting ZEB1/ZEB2, suppressed levels of *miR-200a* may promote EMT. At last, it was observed that *miR-137* and *miR-193a* could be also silenced via hyper-methylation, CDK6 and E2F6 has been suggested as their major targets. It was also shown that *miR-137* hyper-methylation is associated with poorer average survival [74,75].

Urological tumours

Renal cell carcinoma (RCC). The VHL tumour suppressor signalling pathway is the most important deregulated pathway in clear cell RCC, the dominant subtype of kidney cancers. The VHL gene can be spontaneously deleted or hyper-methylated. The regulation of the VHL pathway by miRNAs has not been well studied in RCC. The interactions have been proposed but a direct relation between miRNAs and VHL or HIF1A were not proven. A subset of miRNAs has been identified as regulated by VHL pathway, 3 miRNAs were up-regulated and 6 were down-regulated. The second commonly deregulated pathway is VEGF signalling pathway, which is transcriptionally regulated by HIF (after hypoxia) or due to loss of VHL. Interaction between miRNAs and VEGF has not been well studied in RC, and only *miR-29b* has been shown to indirectly regulate VEGF. However, strong inverse correlation between *miR-200* family and VEGFA has been observed with suggestion that VEGFA is direct target of these miRNAs [76]. Down-regulation of *miR-141* was found in malignant compared to matched non-malignant tissue samples [47].

Bladder cancer. Mutation or over-expression of the FGFR3 gene occurs in approx. 80 % of all patients with low-grade non-invasive urothelial carcinomas. Some well-established de-regulated miRNAs in bladder cancer are predicted to target FGFR3, and regulation of FGFR3 by *miR-99* and *miR-100* of four predicted miRNAs has been experimentally validated. Family *miR-200* is associated with an epithelial phenotype; its ectopic expression in bladder cancer cell lines induces up-regulation of epithelial and down-regulation of mesenchymal markers. Up-regulation of *miR-143* is accompanied by down-regulation of RAS [76]. Transfection of bladder cancer cell lines with *pre-miR-129* exerts significant growth

inhibition and induces cell death. *mir-129* is shown to target GALNT1 and SOX4. Transfection of *miR-30-3p*, *miR-133a* and *miR-199a** results in decrease of tumour cell growth in bladder cancer. *miR-101* inhibits cell proliferation in bladder transitional cell carcinoma by targeting EZH2 and altering global chromatin structure. Down-regulation of *miR-145* in bladder cancer has been also observed [47].

Prostate cancer. A major signal transduction pathway in prostate cancer is PI3K/Akt signalling pathway that is hyper-activated in approx. 30-50 % of prostate cancer. Many of the predicted miRNAs that are predicted to target proteins of this pathway are differentially regulated in prostate cancer. Second pathway is androgen receptor (AR) pathway. *miR-125b* is an androgen-sensitive miRNA, which has been shown to regulate apoptosis through inhibition of BAK1. *miR-101* has been shown to be up-regulated in human prostate cancer and it seems that through inhibition of EZH2 reduce invasion and induce morphological changes in prostate cancer cells [76]. *miR-221* is found to be progressively down-regulated in aggressive forms of prostate cancer. Down-regulation of *miR-221* is linked to cancer progression and recurrence in a high risk prostate cohort. Progressive miR-221 down-regulation also hallmarks metastasis [47,76]. *miR-499a* has been shown to be down-regulated in prostate cancer tissue. Its introduction into prostate cancer cells results in cell cycle arrest and apoptosis, where it regulates cell growth and viability in part by repressing the expression of HDAC1 [47]. *miR-15a-miR-16-1* cluster, located at chromosome 13q14, is deleted in most cases of prostate cancer [21].

Melanomas

Melanoma is the most aggressive type of skin cancer, and it is resistant to therapy in its advanced stages [77]. Abnormalities in several signal transduction pathways, which are important for normal melanocyte development, only partly explain molecular mechanism directly linking UV radiation to the development of melanoma. miRNAs are emerging as important causal factors to melanoma initiation and progression [78]. In 45 primary cultured melanoma cell lines, there was observed that many genomic loci containing miRNAs are frequently affected (85.9 %) by copy number abnormalities. For an example, copy number losses of the region containing *miR-218-1* and SLIT2 were shown in 33 % of all investigated melanoma lines [79]. Proteins involved in miRNA biogenesis, Drosha, Dicer and Ago, are over-expressed in melanoma [32].

However, deregulation of miRNAs expression is not always explained. *let-7* plays a role in melanoma development and progression, its targets are many cancer-promoting molecules, such as NRAS, Raf, c-myc, cyclin D1/D3, and CDK4 [77]. Analyzing 10 melanocytic nevi and 10 primary melanomas, it was revealed that five members of the *let-7* family were significantly down-regulated in melanoma [80]. Over-expression of *let-7b* leads to inhibition of cell cycle progression and inhibition of its targets (cyclin D1/D3/A and CDK4) [77]. A direct interaction of *let-7b* with the cyclin D1 3'-UTR was showed. *Let-7a* was thus demonstrated to regulate expression of integrin beta3 and RAS oncogene, which is highly related to melanoma progression.

Another family, *miR-34*, has tumour suppressive role. Expression of *miR-34a* is silenced due to an aberrant CpG methylation in 43.2 % of melanoma cell lines and in 62.5 % of primary

melanoma samples. The tumour suppressive function of *miR-34a* has not yet been investigated, however, there was shown a reduced expression of *miR-34b/c* and *miR-199a** and it was proposed that their target is MET oncogene [80]. Another miRNA with potentially tumour suppressor role is embedded in CpG island and epigenetically regulated in melanoma, this is *miR-370* [79].

miR-203 has also an important tumours suppressor role in a lot of cancers, and it is often lost due to deletion or due to promoter CpG hyper-methylation. *miR-203* target p63, and their relationship might be relevant also in melanoma, since it is known that *miR-203* functions as switch between epidermal proliferation and differentiation [81].

Gynaecological tumours

miR-125b-1 is located on a fragile site on chromosome 11q24 which is deleted in a subset of patients with ovarian and cervical cancer [61].

Cervical cancer. Cervical cancer aetiology is strongly linked to HPV infection, and involvement of virus protein E6 and E7 in pathogenesis is well established. The exact pathway from infection to tumorigenesis has not been elucidated yet. However, down-regulation was observed for: *let-7b/c, miR-23b, miR-196b, miR-143*, and *miR-145. miR-143* and *miR-145* were equally down-regulated in all cell lines of cervical cancer (HPV infected and HPV not infected), whereas miR-*218* was the unique miRNA down regulated only in HPV-16 and HPV-18 positive cell lines. Down-regulation of *miR-214* is related to the ability of this miRNA to inhibit HeLa cells proliferation through targeting MEK3 and JNK1 transcripts. HPV protein E6 induces destabilization of p53, down-regulation of *miR-34a* and increased proliferation of pre-malignant HPV infected cervical cancer cell lines [82].

Endometrial cancer. Reciprocal association between down-regulation of *miR-192-2* and SOX4 expression was determined; it was further established that restoration of *miR-192-2* induced a decrease in SOX4 expression and this resulted in diminished cell proliferation. Decreased expression for *miR-152* and *miR-101* was found to consist of an independent risk factor for disease free survival. Restoration of those miRNAs by transfection in cell lines lead to diminished cell proliferation. Down-regulation of *miR-101* was correlated with strong positive immunoreactivity of COX2, which was previously shown to be associated with worse prognosis. To date, no data are available for relationship between miRNAs and oestrogen response in endometrial cancer [82].

Ovarian cancer. Inconsistencies are observed between results in ovarian cancer studies for well-known tumour suppressors. These could be due to the differences in study populations and methodologies used, due to the choice of control group and type of control. For instance, the number of studies used as control cell lines and another number of studies used whole normal ovaries. The existence of significant discrepancies in expression profiles of certain miRNAs indicate the need of further and more in-depth research that would establish those results [82].

5.2. lncRNAs as tumour suppressors

lncRNAs are known to mediate epigenetic modifications of DNA by recruiting chromatin complexes to specific loci [8]. Only a handful of lncRNAs have been characterized, and their involvement in control of gene expression [3]. We therefore presented four lncRNAs with proposed tumour suppressor function in cancer.

MEG3

MEG3, located in chromosome 14q32, is maternally expressed imprinted gene, which represents lncRNA, but also hosted miRNAs and snoRNAs. It plays role in cell proliferation, and its expression is under epigenetic control. *MEG3* and its hosted miRNAs and snoRNA could represent a tumour suppressor gene, since aberrant CpG methylation (promoter hyper-methylation, and hyper-methylation of the intergenic region) has been observed in several types of cancer, as well as their gene copy number loss [9].

MEG3 ncRNA might modulate binding of p53 on the promoter of its target genes [9]. It was later verified that *MEG3* was associated with p53 and that this association was required for p53 activation, further suggesting tumour suppressor role for *MEG3*. It was demonstrated that *MEG3* expression is markedly decreased in glioma tissues compared to adjunct normal tissues. Ectopic expression of *MEG3* inhibited cell proliferation and promoted cell apoptosis in glioma cell lines [83]. Growth inhibition is partially due to apoptosis induced by *MEG3*, which induces accumulation of p53, stimulates transcription from p53-dependent promoter and regulates p53 target gene expression. Loss or significantly reduction of *MEG3* expression has been further found in other cancer cell lines examined, bladder, bone marrow, breast, cervix, colon, liver, lung, meninges, and prostate, as well as in other primary tumours, neuroblastoma, hepato-cellular carcinoma, and meningioma. It has been suggested that DNA methylation plays a major role in silencing the *MEG3* gene in tumours [84].

GAS5

LncRNA *GAS5* is highly expressed in cells that have arrested growth and can sensitize a cell to apoptosis by regulating activity of glucocorticoids in response to nutrient starvation. It has been linked with breast cancer. *GAS5* transcript levels are significantly reduced compared to un-affected normal breast epithelia, suggesting that could act as tumour suppressor. *GAS5* maintain sufficient caspase activity to activate appropriate apoptotic response in diseased cells. Chromosomal translocation affecting 1q25 locus that contains the *GAS5* gene has been detected in melanoma, B-cell lymphoma, prostate and breast cancer [7,85]. *GAS5* regulates expression of a critical subset of genes with tumour suppressive consequences [4].

LincRNA-21

LincRNA-p21 is required for the global repression of genes that interfere with p53 function regulating cellular apoptosis; it physically interacts with a protein hnRNP-K, allows it localization to promoters of genes that need to be repressed in a p53-dependent manner [4]. In response to DNA damage, lncRNAs are induced by the p53 tumour suppressor pathway. *lincRNA-p21* plays an important role in cellular response to apoptotic signal, it is induced by p53 and act as an inhibitor of the p53-dependent transcriptional response by repressing the

transcription of genes that interfere with apoptosis (guidance of hnRNP-K to the promoters of genes repressed by p53). *LincRNA-p21* has not been directly associated with disease yet, but loss of function of *lincRNA-p21* might be involved in cancer initiation since functions to trigger cell death through the induction of apoptosis program [7,9,85].

CCND1

It is involved in the regulation of Cyclin D1 gene expression. Cyclin D1 is a cell cycle regulator often mutated, amplified and over-expressed in various types of cancer. After binding of this lncRNA on RNA-binding protein, consequently inhibition of enzymatic activities of the histone acetyltransferases occurs, leading to silencing of cyclin D1 gene. These studies suggest that this lncRNA is a tumour suppressor RNA, which can be rapidly induced by cellular stress to regulate it sense gene expression [85].

6. Conclusion

The rest of ncRNAs, other than miRNAs, in regulation biological functions are more or less unexplored, and this should be further investigated in future research. Regarding therapeutic approaches, we still need more knowledge concerning which miRNAs to target, how to produce and stabilize them, how to direct them to the target tissue. The specificity of drug-like oligonucleotides is important, because of the off-target effect. The off-target effect is also a significant challenge, especially considering that miRNA-mediated repression often requires a homology of only six to seven nucleotides in the seed region of the miRNA and mRNA target site. Toxicity due to chemical modifications, which is used to facilitate cellular uptake and prevent degradation, should be take into account. However, only recently was described the possibility of using exososmes and exosomal tumour-suppressive miRNAs as novel cancer therapy [86].

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Roles of Tumor Suppressor Signaling on Reprogramming and Stemness Transition in Somatic Cells

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Additional information is available at the end of the chapter

1. Introduction

The pioneering landmark, established by Takahashi and Yamanaka (Takahashi et al., 2007; Takahashi and Yamanaka, 2006) in reprogramming somatic cells into induced pluripotent stem (iPS) cells using the four transcriptional factors of Oct4, Sox2, Klf4, and c-Myc, represents one of the most important paradigm shifts in current stem cell biology. This unprecedented discovery could potentially revolutionize regenerative medicine, cell-based therapy and personalized medicine. Despite recent great advancement in cell reprogramming, there are still considerable technical challenges to circumvent restrictions of applications of reprogramming technology (Kawamura et al., 2009; Saha and Jaenisch, 2009). The utilization of over-expressed transcriptional factors, which of many play oncogenic roles, during somatic reprogramming posts the risk of malignant transformation, thus, limiting its clinical applications. Moreover, the reprogramming process using these factors is still inefficient in some of cell types, and is not always successful in other kinds of cells (Kawamura et al., 2009; Marion et al., 2009; Menendez et al., 2012). Therefore, the underlying mechanisms for signaling control of these factors still need to be further explored.

Somatic cell reprogramming is a complicated cellular process that is controlled by many signaling networks. Accumulated evidence indicated that stemness transition can be detected in some tumor cells following the introduction of relevant signal stimulation, and cancer cells or differentiated cells can be changed into stem cell-like cells that go through less-differentiated stages (Chen et al., 2008; Fodde and Brabletz, 2007; Huang et al., 2009; Liu et al., 2009a).

However, stemness transition may not lead to a full reprogramming of treated cells, which is determined by the delicate controls of signaling network activities in living cells. Interestingly, stemness transition may accompany epithelial-mesenchymal transition (EMT) events in cancer cells, and both programs are closely linked to the core stem cell gene network activities. Not surprisingly, multiple signaling pathways have been reported to be involved in EMT events and generation of stem cell-like cells. Wnt/β-catenin and TGF-β signaling are two potent inducers of EMT during embryonic development and cancer progression (Li et al., 2010; Mani et al., 2008; Morel et al., 2008; Scheel et al., 2011). Other involved pathways in these cellular activities may include BMP/Activin/Nodal, Notch, Hedgehog, Fibroblast growth factor signaling, and others (Chen et al., 2008; Huang et al., 2009; Kang and Massague, 2004; Natalwala et al., 2008; Thiery, 2002, 2003; Wu and Zhou, 2008).

The Wnt/β-catenin signaling pathway, highly conserved among various species and composed of a large family of proteins that control many biological properties (Fodde and Brabletz, 2007; Kikuchi et al., 2009; ten Berge et al., 2008b), may play a central role in the control of reprogramming and stemness process. This pathway includes more than two hundred genes and plays a critical role in modulating the delicate balance among stemness, proliferation, and differentiation in certain stem cell niches and tumor cells (Gu et al., 2010; Katoh, 2007; Lowry et al., 2005; Reya and Clevers, 2005). The established evidence reveals that various levels of Wnt/β-catenin signaling are likely to contribute to distinct cellular activities such as stemness transition, differentiation, carcinogenesis, and the EMT program. Therefore, the cellular activities and fate decisions are determined by this signaling activity in both dosage-dependent and tissue-dependent fashions (Anton et al., 2007; Kikuchi et al., 2009; Lluis et al., 2008; Reya and Clevers, 2005; Slack et al., 1995; Tapia and Scholer, 2010a; ten Berge et al., 2008a; Vermeulen et al., 2010). However, whether and how this signaling pathway has its direct influence on pluripotency gene networks and EMT events is largely unexplored.

As mentioned previously, cell fate decisions are controlled by both positive and negative forces in human cells. It has been well-established that tumor suppressor genes (TSGs) are important regulators to control cell proliferation, differentiation and cell death. Not surprisingly, these genes also play important roles in programming, reprogramming, and stemness transition in human cells. The well-studied TSGs, such as *p53*, *p16,* and *RB1*, serve as key regulators for the cell programming (Bonizzi et al., 2012; Hong et al., 2009; Liu et al., 2009b; Marion et al., 2009; Molchadsky et al., 2010; Wenzel et al., 2007). There are a number of reports on *p53* / p21 pathway that are involved in the reprogramming process and stemness transition in somatic cells. It should be noted that Wnt signaling was linked to the *p53* pathway a long time ago, suggesting that both signaling pathways may play interactive and critical roles in cell fate determination (Damalas et al., 1999; Kinzler and Vogelstein, 1996; Lee et al., 2010). Recent findings demonstrated that several mechanisms play a limiting role in somatic reprogramming and cell stemness transition (Figure 1) (Kawamura et al., 2009; Menendez et al., 2012; Menendez et al., 2010; Takahashi, 2010; Tapia and Scholer, 2010b). In most situations, these genes serve as active players or barriers for cell reprogramming. However, many essential questions on the roles of TSGs in cell fate decision remain unclear. For example, whether *p53*-induced inhibition in reprogramming is transient or just in the early stage is still in question (Cox and Rizzino,

2010; Krizhanovsky and Lowe, 2009; Wahl, 2011). Also, it was reported that the loss of *RB1* is critical for the expansion of the stem cell populations (Liu et al., 2009a; Wenzel et al., 2007). Undoubtedly, there is an urgent need to further elucidate the molecular mechanism and signaling pathways in regulating and controlling the process of somatic reprogramming and stemness transition.

Epigenetic regulation is one of the important mechanisms in the regulation of TSG activities. Recently, epigenetic modification has been shown to influence the reprogramming process, suggesting that many known TSGs may be involved in these cellular activities. Some reports illustrated that a dedifferentiation process of somatic cells to iPS cells involves dynamic epigenetic remodeling. In addition, there seem to be interactions between reprogramming transcription factors and epigenetic modifiers during these cellular activities (Takahashi, 2010).

In this chapter, the role of TSGs in cell reprogramming and stemness process, and regulation of these genes during stem cell renewal will be discussed, as described in Figure 1. We will review the role of TSG-mediated pathways and epigenetics as a barrier in cell fate determinations.
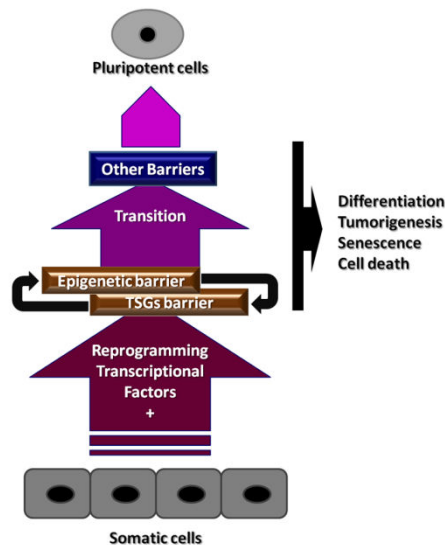
Figure 1. Schematic representative of somatic reprogramming. The reprogramming efficiency is markedly influence by TSG-mediate pathways and epigenetic modifications.

1.1. *CDKN2A* (*p16^{INK4A}* and *p14^{ARF}*) gene

The *CDKN2A* (*INK4/ARF*) locus encodes two important TSGs, the $p16^{INK4A}$ (or *p16*) and $p14^{ARF}$. They are important regulators for two other critical tumor suppressive signaling pathways for controlling cell proliferation, namely *RB1* and *p53*. Utikal et al. reported that secondary murine embryonic fibroblasts (MEFs) were capable of generating iPS cells at early passage, but the

efficiency decreased after serial cell culture passaging and the concomitant onset of cellular senescence (Utikal et al., 2009). This phenomenon was mainly correlated with accumulation of molecular changes in the late passage senescent MEFs (Utikal et al., 2009). Indeed, up-regulation of $p16^{INK4A}$ (*INK4A*), $p14^{ARF}$ (*ARF*), and $p21^{CIP}$ was concurrently observed in the late passage of the MEFs (Utikal et al., 2009). Deficiency and knockdown of *INK*, *ARF*, and *p53* expression resulted in higher efficiency of iPS cell formation. Interestingly, when MEFs were cultured in low oxygen condition (4%), both the expression of *INK4A* and *p53* were reduced. Most importantly, the efficiency of the iPS reprogramming was increased in the low oxygen condition. This further supports the role of *CDKN2A* and *p53* in inhibiting the reprogramming process (Utikal et al., 2009).

Concurrently, Li et al. also worked on the role of *INK4/ARF* locus which encodes three TSGs, $p16^{IN4A}$, $p14^{ARF}$, and $p15^{INK4B}$ on the reprogramming of differentiated cell into iPS cells. They showed that the locus is completely silenced in iPS and embryonic stem cells. The three transcription factors, Oct4, Klf4, and Sox2 repressed the gene expression of $p16^{INK4A}$, $p14^{ARF}$, and $p15^{INK4B}$ with concomitant appearance of iPS cells. In addition, genetic knockdown of the *INK4/ARF* locus improved the efficiency of iPS cell generation. In mouse cells, *ARF* played more significant role as compared to *INK4A*. In contrast, the *INK4A* function was more prominent than the *ARF* in human cells (Li et al., 2009). Interestingly, ageing up-regulated the gene expression of the three genes at the *INK4/ARF* locus and, in turn, led to less efficient reprogramming in cells from old organisms; this defect can be rescued by genetically inhibiting the *INK4/ARF* locus. Taken together, these findings provide strong evidence that supports the role of *CDKN2A* in regulating cell reprogramming in iPS cells.

The epidermis is a tissue that undergoes continual and rapid self-renewal, and which is dependent on the presence of stem cells and transient amplifying keratinocytes. In primary human keratinocytes, *INK4A* also plays an important role in regulation of their stemness properties (Maurelli et al., 2006). The *INK4A* inactivation enabled the primary human keratinocytes to escape replicative senescence and blocked clonal evolution and maintained keratinocytes having the stemness phenotypes. A persistent *INK4a* inactivation is necessary for maintenance of immortalization of the keratinocytes, which was accompanied by reactivation of B cell-specific Moloney murine leukemia virus site 1 *(Bmi-1)* expression and telomerase activity. *Bmi-1* expression is necessary to maintain the immortalization induced by *INK4a* inactivation. In contrast, the *INK4a* inactivation in the transient amplifying keratinocytes did not undergo immortalization but senescence. Thus, *INK4a* inactivation appears to selectively inhibit clonal conversion in highly proliferative somatic cells. Interestingly, inactivation of *INK4a* up-regulated the $ARF/p53/p21^{Waf1}$ pathway but this up-regulation of the *p53* pathway was unlikely to suppress the cell proliferation. The *p53* pathway was necessarily inactivated during immortalization of human keratinocytes. This study clearly indicates the regulation of keratinocyte clonal evolution by *INK4a* regulation and its inactivation in epidermal stem cells is necessary for maintaining the stemness phenotypes (Maurelli et al., 2006).

1.2. *RB1* gene

RB1 (*pRB1* family members: *RB1*, *RBL1*, and *RBL2*) was identified as a TSG in patients with inherited retinoblastoma. It is one of the well-studied TSGs. It involves in cell cycle G_1/S transition regulation and binds to an important transcription factor family, E2F. Based on the Knudson two-hit hypothesis, loss of single copy of *pRB1* gene is not sufficient to induce tumor formation, loss of another copy is necessary for inducing tumor formation (Knudson, 1971). Mouse *pRB1*was found to be crucial during embryonic development; loss of two copies of *RB1* gene in mouse embryo is lethal (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992; Wu et al., 2003). Trophoblasts are cells forming the outer layer of a blastocyst, which provide nutrients to the embryo and develop into a large part of the placenta. Specific loss of mouse *pRB1* gene in trophoblast stem cells resulted in an overexpansion of trophoblasts, profound placental abnormalities, and eventually fetal death (Wenzel et al., 2007). Loss of *pRB1* resulted in an increase of *E2F3* expression and the combined depletion of *pRB1* and *E2F3* in trophoblast stem cells rescued the *pRB1* mutant phenotypes by restoration of placental development and by extending the lifespan of embryos. As can be seen, the *pRB1* pathway plays a critical role in the maintenance of a mammalian stem cell population for proper development of both extra-embryonic and fetal tissues.

Humans and other mammalians are unable to regenerate large portions of lost limbs or other internal organs after traumatic injury or surgical excisions. In contrast, lower vertebrates are able to regenerate entire limbs, the lens of the eye, and portions of the heart (Brockes and Kumar, 2008; Poss et al., 2002; Tanaka and Weidinger, 2008). The difference can be explained in part by the observation that inactivation of *pRB1* alone in lower vertebrates was sufficient to induce skeletal muscle regeneration by reversing differentiation and post-mitotic arrest in the muscle cells (Tanaka et al., 1997). In mammalian muscle cells, suppression of *pRB1* alone was not sufficient to reverse the post-mitotic arrest and terminal differentiation (Camarda et al., 2004; Huh et al., 2004; Pajcini et al., 2010). The tumor suppressor *ARF* which is present in mammals, but absent in regenerative vertebrates, is a regeneration suppressor in addition to *pRB1* (Pajcini et al., 2010). Concurrent inactivation of both *ARF* and *pRB1* resulted in mammalian muscle cell cycle re-entry cell proliferation and dedifferentiation (Pajcini et al., 2010). These results indicate that suppression of both *pRB1* and *ARF* will result in the ability of skeletal muscle cells to lose their differentiated characters, and the skeletal muscle cells will then proliferate and dedifferentiate in a manner that mimics the regenerative lower vertebrate cells. Furthermore, *pRB1* is not only restricted to serve as a cell cycle regulator, but also to impact differentiation and tissue-specific gene expression directly by binding histone deacetylase 1 (HDAC1) and promoting activation of muscle genes such as the myogenic activator MyoD (Puri et al., 2001).

The *pRB1* gene family plays an important regulatory role in neuronal differentiation (Slack et al., 1995). When treated with retinoic acid, the embryonal carcinoma p19 cells were induced to differentiate into cultures primarily consisting of neurons and astrocytes. During this neuroectodermal differentiation, a dramatically increase of pRB1 protein levels was observed. When the pRB1 family proteins in the p19 cells were inactivated by the E1A mutant, the differentiating p19 cells underwent apoptosis. The dying cells were those committed to the

neural lineages because neurons and astrocytes were lost from the differentiating cell culture. The results suggest that the pRB1 family proteins are essential for the neural lineage development and the absence of functional pRB1 activities will trigger cell death of the differentiating neuroectodermal cells.

The *pRB1* pathway is also critical for inducing the cellcycle arrest that mediates cell-cell contact inhibition in fibroblasts; when all three *pRB1* family members, *RB1*, *RBL1*, and *RBL2*, were inactivated by triple knockouts (TKOs), the fibroblasts escaped from contact inhibition and grew into 3D colonies or stacks in cell culture (Dannenberg et al., 2000; Sage et al., 2000). The outgrowth of TKO MEFs into spheres triggered reprogramming to produce cells with cancer stem cell properties. Whereas the fibroblasts with a single *pRB1* mutation retained contact inhibition, when this inhibition was bypassed by forcing the cells to form outgrowth spheres, the fibroblasts were reprogrammed to generate cells with a cancer stem cell phenotype (Liu et al., 2009a). These findings suggest a potential mechanism for generation of cancer stem cells from differentiated somatic cells as a result of tumor outgrowth.

1.3. *p53* gene

p53, as the "guardian of the genome" (Lane, 1992), plays a pivotal role in regulating the delicate balance of cell proliferation and cell death (Molchadsky et al., 2010). Since its discovery more than three decades ago, the role of *p53* in suppressing tumor initiation and progression is well established. It is, therefore, not surprising that *p53* is lost, inactivated, or mutated in the majority of cancers. In respond to external stress stimuli, *p53* prevents cancer development by inducing cellcycle arrest, DNA repair, senescence, and apoptosis.

Researchers have newly identified roles played by *p53* including regulation of pluripotency and dedifferentiation, as a potent barrier in reprogramming. (Hong et al., 2009). Undoubtedly, the function of *p53* is now far more complex than just simply playing the role as the classical tumor suppressor (Bonizzi et al., 2012; Kawamura et al., 2009; Marion et al., 2009; Menendez et al., 2010; Molchadsky et al., 2010; Tapia and Scholer, 2010a; Wahl, 2011; Zhao and Xu, 2010). This provides us with a new insight on the complexity of *p53* signaling in controlling cell fate decisions. Despite accumulating effort in deciphering the diversified roles played by *p53*, the cellular and molecular mechanism underlying the acquisition of "stemness" involved in the *p53* signaling is still largely unexplored.

During somatic cell reprogramming, the *p53* pathway is activated, thus disrupting iPS reprogramming (Kawamura et al., 2009). *p53* may act as a limiting factor in the iPS reprogramming efficiency. Inhibition of the *p53* pathway either by mutating, deleting or knocking down *p53* or its target gene , *p21*, further enhances the reprogramming efficiency (Kawamura et al., 2009; Liu et al., 2009b; Marion et al., 2009; Tapia and Scholer, 2010b).

The *p53*/*p21* pathway was reported to suppress the iPS cell generation. Suppression of *p53* increased the efficiency of the generation of iPS cells (Hong et al., 2009). A dominant negative *p53* mutant, P275S, was used to study the effect of *p53* on regulating the iPS cell generation. Results suggested that inhibition of *p53* function by introducing the dominant negative *p53* mutant into the MEFs increased GFP-positive colonies in the *p53*-heterozygous MEFs (Hong

et al., 2009). Similar experiments were also performed in terminally differentiated somatic cells (T-lymphocytes from Nanog-GFP reporter transgenic mice with either *p53* wild-type or null genotype). In this study, the four important stem cell reprogramming factors, *Oct4, Sox2, Klf4,* and *c-Myc* were introduced into the T-lymphocytes. No GFP-positive colony can be observed in the *p53* wild-type T lymphocytes (Hong et al., 2009). On the other hand, GFP-positive colonies can be observed in *p53*-null lymphocytes and the cells were expandable and have a similar morphology with the mouse ES cells (Hong et al., 2009). The increased GFP-positive cells can also be observed in the adult human dermal fibroblasts (HDFs) by introducing the dominant negative *p53* together with the reprogramming factors into the HDFs (Hong et al., 2009), suggesting the importance of *p53* in regulating the iPS cell reprogramming.

The function of *p53* in regulating stem cell multipotency was confirmed in germ-line stem cells (GSCs). Depletion of *p53* function in the GSCs increased the efficiency of reverting GSC multipotency status (Kanatsu-Shinohara et al., 2004). This finding can also be observed in a *p53* knockout mouse study (Lam and Nadeau, 2003). Hanna et al. suggested that depletion of *p53* function in clonal B cells can only enhance the kinetics of reprogramming somatic cells into iPS cells with a higher cell division rate (Hanna et al., 2009). However, it does not regulate the overall efficiency (Hanna et al., 2009). A *p53* mutant, R172H, which induces conformation change of the *p53* protein, was reported to associate with higher reprogramming efficiency than WT *p53* in the MEFs (Lang et al., 2004). Lang et al. showed that reprogramming efficiency in that particular *p53*-mutated MEFs, which was induced by utilizing a two factor system (Oct4 and Sox2), is higher than the *p53* knockout MEFs that was induced by using the three factors system (Oct4, Sox2, and Klf4) (Lang et al., 2004), suggesting the importance of *p53* in regulating the reprogramming process.

Cicalese et al. suggested that the function of *p53* in stem cells is critical to maintain a constant number of stem cells by imposing an asymmetric mode of self-renewing division. In the *p53*$^{-/-}$ and *ErbB2* tumor mammospheres, up-regulation of Nanog is observed. These studies also revealed the importance of *p53* in regulating the stem cell polarity, and the loss of *p53* induces increased frequency of symmetric division and tumor initiation and growth (Cicalese et al., 2009).

The suppression of the reprogramming efficiency of iPS cells by *p53* can be associated with the maintenance of genomic integrity of iPS cells. Deficient *p53* resulted in shorter telomeres in the reprogramming MEFs (Marion et al., 2009), suggesting the low efficiency of reprogramming in the WT *p53* cells to prevent the spreading of cells upon DNA damage and to ensure iPS cell genomic integrity (Marion et al., 2009).

Another barrier affecting the reprogramming is the *INK4A/ARF* tumor suppressor locus, as described previously. A recent report by Li and colleagues illustrated that the *INK4A/ARF* locus was suppressed during the early stage of reprogramming, leading to inactivation of the *p53* and *pRB1* pathways (Li et al., 2009). Interestingly, cells with *p16*[INK4A] knockdown alone are sufficient to enhance the reprogramming efficiency (Li et al., 2009). Together, these observations indicate that both *p53* and *pRB1* may work synergistically as barriers in somatic cell reprogramming (Li et al., 2009; Menendez et al., 2010; Utikal et al., 2009).

In a recent report by Lee K.H. et al., *p53* preferentially targets the Wnt signaling pathway in the murine ESC differentiation program (Lee et al., 2010). Evidently, the crosstalk between *p53* and Wnt signaling pathway plays an integrated role in stemness acquisition. A *p53* downstream phosphatase, *Wip1*, which shows high expression in the intestinal cells, was reported to associate with *p53*-dependent apoptosis of stem cells in the mouse intestine (Demidov et al., 2007). Removal of *Wip1* reduced the polyp formation in the *APC*^Min mice. The *APC*^Min/+ mice contain a nonsense mutation in the *APC* gene. Constitutively activated Wnt signaling pathway increased the apoptosis events of intestinal stem cells in the *Wip1*-deficient mice (Demidov et al., 2007). Low level of *Wip1* reduced the threshold of *p53*-dependent apoptosis of stem cells. However, *Wip1* deficiency does not affect the activities of β-catenin in terms of its nuclear localization level. A high level of β-catenin can be observed in the nuclei of polyp cells and this contributes to up-regulation of *c-Myc* and *Cyclin D1* in the *Wip1* null/*Apc*^Min/+mice. The β-catenin signaling pathway activation and attenuation of *p53* resulted in increasing efficiency of intestinal stem cell apoptosis (Demidov et al., 2007).

Recently, researchers demonstrated that the p53-miR34-Wnt network is a determinant factor of dichotomy between stem cell properties and tumor progression. miR34, one of the direct downstream targets of *p53*, is found to interact with Wnt and EMT genes, including *β-catenin, AXIN2, LEF1* and *Snail*. With the loss of *p53* due to miR34, the Wnt pathway is activated, which further induced the transformation of EMT (Liu et al., 2011). Therefore, the *p53* gene plays an important role in the controlling EMT.

Chang et al suggested that *p53* induced transcriptional activation of microRNA, miR-200c, through direct binding to its promoter region. The miR-200c was reported to regulate the EMT process through inhibition of transcriptional suppressors of an epithelial marker, E-cadherin (Chang et al., 2011). The miR-200c can target to and suppress ZEB1/2 (Wellner et al., 2009), which is a well-studied E-cadherin transcriptional suppressor and thus, regulates the EMT process. The knockdown of *p53* in MCF12A cells resulted in loss of epithelial phenotype and shows a significant elevation of the CD24^-CD44^+ population. Re-expression of *p53* in TGF-β-treated MCF12A showed inhibition of TGF-β-induced increase of the stem cell population.

p53 is not a sole player in deciding the cell fate determination. In fact, *p53* works as an integrated network, interplaying with other important pathways, depending on the external stimuli and microenviroment. However, there is a great need to further elucidate the roles of the *p53* network in reprogramming, dedifferentiation, self-renewal, and pluripotency.

2. Signaling pathways involved in the reprogramming and stemness transition

2.1. TGF–β signaling pathway

TGF-β signaling pathways play multiple roles in regulating tumorigenesis and other cellular processes, including reprogramming, stemness transition, and EMT events. Many components in this signaling pathway were defined to participate in both oncogenic and tumor suppressive pathways in various tumors. This provides a complicated story for researchers to study the

function of TGF-β signaling pathways in stem cells or reprogrammed cells. The ligands of the TGF-β family have multiple functions and can cause opposite effects in different cell types. The TGF-β can regulate cell proliferation, growth arrest, differentiation, survival, cell migration, and also the pluripotency of cells. In cancer, over-expression of TGFβ1 and deregulation of the TGF-β receptor type II (TGFBRII) were reported to associate with skin cancer tumorigenesis and invasiveness (Cui et al., 1996). However, the role of TGF-β signaling in regulating reprogramming is still not well-defined. In a previous report, TGF-β family ligands play an important role in reprogramming of somatic cells into iPS cells, regulating ESCs self-renewal, pluripotency maintenance, and controlling differentiation.

TGF-β signalling may have the ability to induce reprogramming of somatic cells into iPS cells. Treatment of TGF-β/activin inhibitor in partially reprogrammed iPS cells can induce Nanog expression (Ichida et al., 2009; Maherali and Hochedlinger, 2009). Furthermore, the functional role of TGF-β in regulating the reprogramming was defined by utilizing chemical TGF-β inhibitors. Interestingly, inhibition of TGF-β signaling can enhance the mouse fibroblast reprogramming efficiency. A substitute of Sox2 (E-616452) and TGFBR1 kinase (SB-431542) inhibitor, were reported to replace the function of Sox2 in MEFs with Oct4, Klf4, and c-Myc expression (Ichida et al., 2009; Maherali and Hochedlinger, 2009). These results suggest the important roles TGF-β plays in the controlling reprogramming process.

Maintenance of the pluripotencies and self renewal properties are important for both ESCs and iPS cells.The canonical TGF-β signaling pathway may play important regulatory roles in ESCs maintenance and generation of pluripotency. BMP4 together with the LIF protein can induce Oct4 expression (Ying et al., 2003). The BMP activated Smad signaling to support self-renewal properties of stem cells. The inhibition of Smad activities by the Smad6 and Smad7 in the ES cells induced smaller and fewer ES cell colon formation (Ying et al., 2003). Secretion of BMP4 by the feeder cells is necessary for ES cell self-renewal (Ying et al., 2003). Inhibition of the Erk and p38 MAPK pathways can further enhance the BMP4-associated effect on self-renewal of mouse ESCs (Qi et al., 2004). Besides this, bFGF (basic fibroblast growth factor) and activin are also important to maintain the pluripotency in human ESCs (Greber et al., 2010; James et al., 2005). The TGF-β signalling may play multifunctional roles in regulating pluripotency of cells. Smad1 was reported to suppress the expression of Nanog by inhibiting its promoter activities (Jiang and Ng, 2008; Xu et al., 2008). The Smad proteins were reported to bind directly to Nanog promoter (Xu et al., 2008) and this is the major mechanism for Smad proteins to regulate Nanog expression. These results suggest the multiple roles of TGF-β signaling in the regulation of stem cell renewal.

Furthermore, TGF-β also plays a role to control the differentiation of ESCs. One of the TGF-β family members, BMP4, was reported to associate with induction of inhibitor of differentiation (*Id)* gene via interaction with the LIF/Jak-Stat3 and Smad pathways. The *Id* gene is an important factor to block ESC differentiation. The undifferentiated ES cells expressed BMP signaling ligands (Ying et al., 2003) and regulated downstream molecules, the Smads, to control the cell differentiation process (Ying et al., 2003).

Collaborating with Wnt signaling, TGF-β signaling is also involved in the EMT program and both pathways are regarded as the axis of EMT in breast cells (Scheel et al., 2011). The hy-
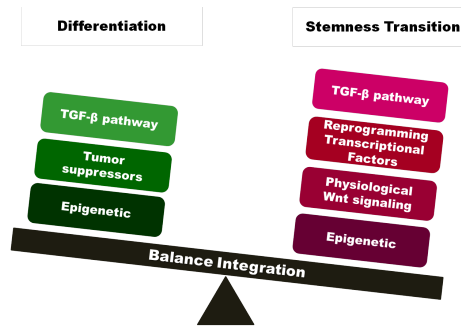
Figure 2. The cell fate determination is delicately controlled by positive and negative forces. Cellular activity balance regulated by both core stem gene-mediated pathways and TSGs is the key determinant in reprogramming process.

pothesis of these two pathways linked to the stem cell networks and TSG pathways is presented in Figure 2.

2.2. Wnt pathway

Cellular reprogramming can be achieved by overexpression of defined transcription factors in somatic cells (Ichida et al., 2009; Takahashi et al., 2007). However, the underlying mechanism of signaling activities that regulated these factors are not fully understood now. Overexpression for certain genes may not be suitable for all pathways, such as *β-catenin*, a mediator of Wnt signaling, because discrete levels of expressed genes are usually needed for maintaining the pluripotent status or direct programming through this pathway (Gu et al., 2010; Lluis et al., 2008; Marson et al., 2008; Merrill, 2008). It still remains unclear the gene-dosage effects of critical factors on somatic cell reprogramming and stem cell renewal. Recent studies revealed that activation of Wnt/β-catenin signaling may directly control reprogramming of fused somatic cells. For example, Wnt stimulators, Wnt3a and BIO, strikingly enhanced reprogramming ability after cell fusion (Lluis et al., 2008; Merrill, 2008). The fusion clones derived from both ESCs and somatic cells had an obvious β-catenin accumulation with increased expression of *Axin2*, a *β-catenin*-dependent gene, suggesting that basic or lower levels of stabilized β-catenin might drive somatic cell reprogramming.

The lower levels of Wnt signaling play a critical role in the control of development of several types of tissues through a dosage-dependent manner, as reported in crypt progenitor cells (Batlle et al., 2002; Korinek et al., 1998), hair follicles (Lowry et al., 2005), and hematopoietic stem cells (Luis et al., 2011). Taken together, observations from both *in vitro* and *in vivo* studies indicated that Wnt/β-catenin signaling was a single dominant force in the control of cell fate determinations in some of tissues, which suggests that basic or physiological levels of Wnt signaling may be required for many cellular activities.

More and more evidence revealed that Wnt signaling plays important roles in maintenance of pluripotency in ESCs and cell self-renewal (Cole et al., 2008; Lluis et al., 2008; Macarthur et al., 2009; Marson et al., 2008; Takao et al., 2007). For example, expression of *β-catenin* was confirmed

to associate with hemtopoietic stem cells and neural stem cell growth (Kalani et al., 2008; Reya et al., 2003). *Wnt3A* activation associated with expression of the stem cell reprogramming markers, Oct4 and Nanog (Ogawa et al., 2007) and maintenance of the pluripotency of mouse ES cells (Hao et al., 2006; Singla et al., 2006). *Wnt3A* induced generation of iPS cells in the absence of *Myc* (Marson et al., 2008). Those cells contained iPS cell properties and were able to form teratomas during subcutaneous injection into SCID mice (Marson et al., 2008). The Wnt signaling pathway is also involved in regulating pluripotency factors, *Oct4*, *Nanog*, and Sox2 expression (Anton et al., 2007; Sato et al., 2004). This observation was confirmed by down-regulation of the stem cell pluripotency genes in the *β-catenin* deficient mouse ES cells (Anton et al., 2007). Wnt signaling pathway was also associated with cell reprogramming through the telomerase reverse transcriptase (TERT) and Brahma-related gene 1 (BRG1) interaction (Barker et al., 2001) to modulate chromatin structure during reprogramming (Miki et al., 2011).

Interestingly, previous study demonstrated that Wnt3a can also stimulate human ES cell differentiation, rather than only regulate human ES cell proliferation. The canonical Wnt signaling levels are minimal in the undifferentiated human ES cells but greatly increase after Wnt3a treatment and induce differentiation (Dravid et al., 2005). Dramatic increase of reprog-rammed cell numbers can be observed when ES cells, which have a low level of nuclear β-catenin, are fused with neural stem cells. This is mainly due to the low nuclear β-catenin level being able to protect fused cells from apoptosis (Lluis et al., 2010), suggests the importance of β-catenin levels in the regulation of stem cell reprogramming. This finding may help to explain the balance between the maintenance of pluripotency of stem cells and apoptosis, as excess β-catenin can induce p53 expression (Damalas et al., 1999), which was found to induce apoptosis in stem cells to maintain genome integrity. The p53 protein was reported to be a transcription regulator of the Wnt signaling and it bound on the promoter regions of some Wnt signaling members for a general stress response in the mouse ES cells (Lee et al., 2010), which may provide a feedback mechanism to control the deregulation of the β-catenin during the reprogramming process.

It should be noted that inappropriate activation of components of this signaling pathway have been observed in many human cancers and differentiated stem cells, in which the high levels of *β-catenin* signaling were usually detected (Dravid et al., 2005; Fodde and Brabletz, 2007; ten Berge et al., 2008a; Vermeulen et al., 2010). Except for *p53* described previously, some compo-nents of the Wnt pathway can be regarded as both oncogenes and TSGs. For example, *AXIN2*, *APC*, *DKK1*, and *WIF1* are negative regulators of this pathway, and are called TSGs. In summary, the detailed mechanism of Wnt signaling in the control of stemness transition and reprogramming of somatic cells needs to be further explored.

3. Possible mechanisms to regulate TSGs expression in reprogramming

It is well-accepted now that epigenetic regulations are important events to control gene expression in human cells. Promoter hypermethylation and histone modification are two major events to regulate gene expression in various human tumors. The *DNA methyltransferase*

(*DNMTs*), *histone deacetylases* (*HDACs*), *histone acetyl transferase* (*HATs*), and *histone methyl-transferase* are the key regulators to controlgene expression in the genome. Epigenetic changes of gene expression were reported to be important during the iPS cell reprogramming (Han and Sidhu, 2008). The epigenetic changes can also help to maintain the pluripotency by regulating the expression of the key transcription factors, Oct4, Nanog, and Sox2 (van Vlerken et al., 2012). In previous studies, mouse ES cell genomes were found to contain less methylation than the somatic cells, while human ES cells show a distinct epigenetic profile, when compared to somatic cells (Jackson et al., 2004; Lagarkova et al., 2006; Zvetkova et al., 2005). A silenced TSG, *p16*, was found to be re-expressed during the reprogramming process (Ron-Bigger et al., 2010). On the other hand, a previous study suggested that the promoter region of INK4A/ARF was found to be hypermethylated in the iPS and ES cells. Inhibition of *DNMTs* by inhibitor and siRNA increased the *INK4A* and *p21* (*CIP1/WAF1*) expression in human umbilical cord blood-derived multipotent stem cells (So et al., 2011). However, the epigenetic regulation of TSGs during the reprogramming process is still not fully understood now. It is necessary to further explore epigenetic changes of TSGs in the reprogramming process and relevant other cellular activities.
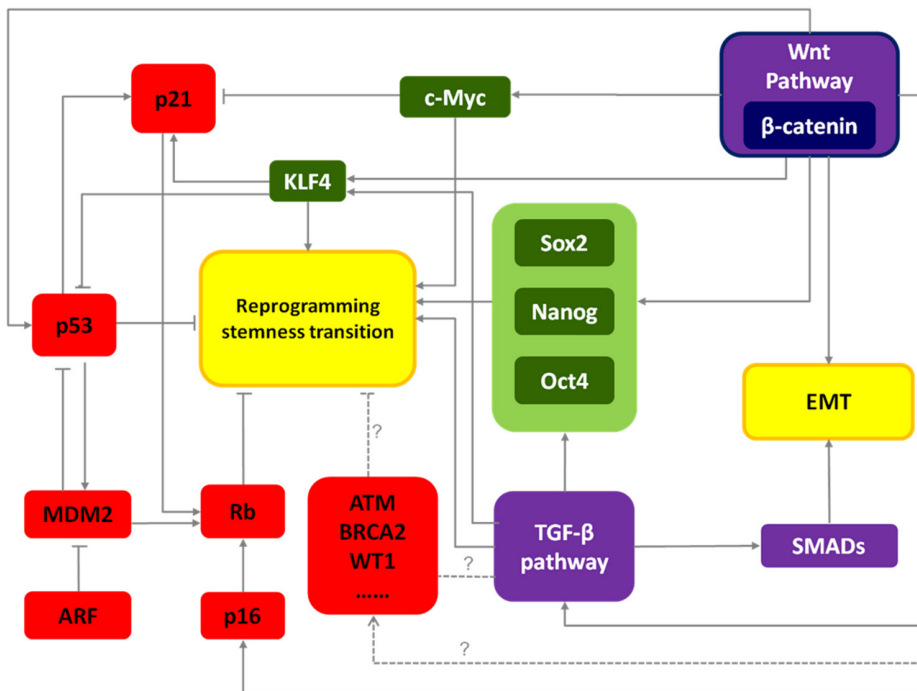
Figure 3. Hypothesis of integrated networks of TGSs, Wnt/β-catenin and TGF-β pathways in controlling reprogramming, stemness transition and EMT events. These pathways may play central roles in regulating other TSGs, transcriptional factors and other signaling pathways.

4. Conclusions

The known and unknown TSGs are the important participators in the regulation of cell reprogramming and stemness transition. These genes are components of various signaling pathways, and play different roles in maintaining cell pluripotency, regulating cell differentiation and proliferation, cell cycle control, apoptosis, and other cell fate decisions. These genes controlling cellular activities act in a time-dependent or a dosage-dependent manner in various tissues. Although detailed underlying mechanisms are not fully clear now, more and more evidence indicates that some TSG signaling activities are determinant forces in important cellular processes, including cell reprogramming. A proposed hypothesis illustrates this in Figure 3. Understanding of the delicate control of these signaling networks in living cells will provide more insight in reprogramming studies and regenerative medicine.

Acknowledgements

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Modeling Tumorigenesis in *Drosophila*: Current Advances and Future Perspectives

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Additional information is available at the end of the chapter

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1. Introduction

1.1. Tumor suppressor genes, a historical perspective

Cancer is essentially considered to be a genetic disease caused by the accumulation of multiple genetic or epigenetic lesions in tumor-suppressor genes and oncogenes [1]. Although the notion that retinoblastoma could be an inherited disease was already formulated at the end of the 19th Century, a solid genetic basis was established with the discovery of both proto-oncogenes, whose gain-of function mutations or altered expression is associated with the cancerous state, and tumor suppressor genes (TSGs), whose inactivation releases the "brakes" inhibiting cell proliferation. Analysis of both proto-oncogenes and TSGs revealed also that cancer results from an alteration of the normal pathway of cell fate and differentiation. The hallmarks of cancer, as laid down by Hanahan and Weinberg to explain the complex biology of cancer, comprise of six major developmental changes taking successively place in human tumors. These cancer "characteristics" include sustained proliferative signaling, evasion of growth suppressors, resistance to cell death, replicative immortality, angiogenesis as well as cell invasion and metastasis. Underlying these hallmarks are genome instability, inflammation, reprogramming of energy metabolism and evading immune destruction [2].

Cancer cells are the foundation of the cancer disease, as they initiate formation of tumors and drive tumor progression forward. Based on the sequence of events in which cells accumulate genetic lesions, tumor progression and metastasis are highly variable, even among tumors of the same type [3]. Previously, cancer cells within tumors were thought to be largely made of homogenous cells until relatively late in the course of tumor progression, when hyperprolif-eration combined with increased genetic instability spawn distinct clonal populations. Now we know that tumors rather than homogenous masses of proliferating cancer cells are complex

tissues composed of distinct cell types, participating in heterotypic interactions with each another. Reflecting such clonal heterogeneity is the finding that many human tumors display a complex histological pattern, characterized by regions exhibiting various degrees of differentiation, proliferation, vascularity, inflammation and invasiveness [2]. In addition, tumors exhibit another dimension of complexity arising from the surrounding normal cells of the "tumor microenvironment" [2] (analyzed in part 3).

Over the last decades the origin of oncogenesis has been the subject of different theoretical "fashions". In the current view taking into account the role of oncogenes and tumor suppressor genes, cancer results from a failure occurring more in the control of cell differentiation than in cell proliferation [4, 5]. Nowadays, cancer is generally considered to result from a block or an error in the normal progression of differentiation. As a result, the cancer cells escape the mechanisms controlling normal growth and proliferation. Several decades ago, pioneer studies in the field of *Drosophila*, mouse somatic cells and human genetics revealed that neoplasia may result from a loss of function in regulatory genes controlling cell growth and differentiation [6-9]. In the following years, research in developmental biology has greatly contributed to cancer research. Indeed very often the initiating event in the formation of a malignant tumor is a block of a critical step in normal differentiation, usually through inactivation of a single gene, and can be accompanied by events occurring in parallel. In the case of tumor-suppressor genes, proliferation of tumor cells is suppressed by the same set of genes that suppress the proliferation of normal cells, in the same cell type during the process of differentiation. Studies of the *Drosophila lethal [2] giant larvae (lgl)* gene, the first cloned tumor suppressor gene, have shown beyond doubt that tumors can be produced at a defined period of development by the impairment of a gene that normally regulates a critical step of differentiation [5, 10, 11]. At that time, such precise time delimitation in the process of development would not have been possible to achieve with mammalian cells, but the observations made thereafter in mammals were consistent with the conclusion derived from the *Drosophila* study.

1.2. Identification of the first tumor suppressor genes in Drosophila melanogaster

Over the past 40 years it has become increasingly evident that cancer is causally related to mutations in specific genes. These genes are instrumental to developmental processes such as cell-cell communication, signal transduction, regulation of gene expression, translation, cytoskeletal organization, protein folding and transport, and differential regulation of cell cycle [12]. The *Drosophila* field has made marked contributions in many of the mechanisms that are fundamental to cancer processes, several of which have been later validated in vertebrates. Less well known is the precursor role of *Drosophila* in the cancer field, as some of the earliest tumor suppressors were identified in flies. The first tumor-containing mutant was recognized in 1967 in a wild type laboratory stock of *Drosophila melanogaster* [6]. The mutant gene was soon identified by Ed Lewis as an allele of the already known *lgl* gene, which was discovered by Bridges in 1933 [13]. During the 1940-50s, this gene has been the subject of a number of developmental studies performed by Hadorn and his collaborators. The phenotypic studies performed by Hadorn's group on *lgl* and other *Drosophila* genes have greatly contributed to the conceptual basis of developmental genetics. In 1955 Hadorn published in German

his seminal monography on "*Lethalfaktoren und ihrer Bedeutung für Erbpathologie und Genphy-siologie der Entwicklung*" which in 1961 was translated into English as "*Developmental Genetics and Lethal Factors*". Comparative developmental studies conducted thereafter showed that one of the pleiotropic effects of the mutation was the formation of a malignant neuroblastoma in the larval brain and the appearance of imaginal disc tumors [7, 14]. Molecular studies on the *lgl* gene were initiated in 1985 by Mechler and his co-workers by cloning the first tumor suppressor gene [10]. Subsequent analysis of *lgl* has demonstrated unequivocally that the tumorous phenotype results from the lack of *lgl* function and showed that tumorigenesis can be rescued by reintegrating a wild type copy of *lgl* into the genome of *lgl*-deficient animals [15]. Biochemical and genetic analysis of the *lgl* gene and its human homologue *hugl-1*, showed that the encoded proteins are components of the cytoskeleton and interact physically with a domain located near the carboxyl terminal of the non-muscle myosin II heavy chain [11, 16-20]. Further studies also revealed that the Lgl protein can interact with the Nucleosome Assembly Protein-1 (NAP-1), a component of the cyclinB-p34^{cdc2} kinase complex [21, 22] and NAP-1 is intimately associated with the cytoskeletal matrix during interphase and accumulates in the nucleus during prophase where it becomes associated with the spindle apparatus [21].

Recent contributions show that the Lgl protein may directly contribute to genetic regulation in association with the heavy chain of nonmuscle myosin II, or nmMHC II [23, 24]. In particular mutations in *lgl* or heteroallelic combinations between *lgl* and *zipper*, encoding nmMHC, were found to block the disintegration of the salivary glands by blocking the program induced by the molting hormone ecdysone [23]. An interaction between both proteins was found to be required for the binding of specific nuclear remodeling proteins to chromatin [24]. Defect in this interaction may result in a block of genetic cascade initiated by the ecdysone hormone and lead to a transcriptionally frozen genome. The outcome of these analyses shed light on the key roles that tumor suppressor genes may play not only in the mechanism of cell shape and tissue organization but also in the regulation of developmental programs.

Subsequently to these initial studies, Gateff isolated a series of other recessive mutations in distinct genetic loci, which gave rise to four specific types of tumors. These tumors affected either the developing larval brain, the imaginal discs, the hematopoietic organs, or the germ cells [25, 26]. Shortly after *lgl* was reported [6], a genetic screen assaying imaginal disc morphology identified a mutation in the *discs large* (*dlg*) gene, coding for a septate junction tumor suppressor gene [27] with a second mutation identified few years later [28]. Twenty years later, a third mutation with the strikingly similar phenotypes, called *scribble* (*scrib*), was independently isolated in two different labs. Initially *scrib* was found in a screen for regulators of epithelial architecture [29]. Parallel to this investigation, a P-element mutagenesis screen led to the identification of the recessive *scrib^{vartul}* mutation causing late larval lethality, imaginal disc overgrowth and brain tumors with a complex syndrome reminiscent of that observed in mutations of the other tumor suppressors [30]. Therefore, already at the very early days of tumor genetics, *Drosophila* has been an extremely favorable object of study. Since then a great number of tumor suppressor genes have been identified and *Drosophila* has largely contributed to our understanding of the basic biology and cellular mechanisms of tumorigenesis including cell growth and proliferation, apoptosis, maintenance of cell polarity and architecture.
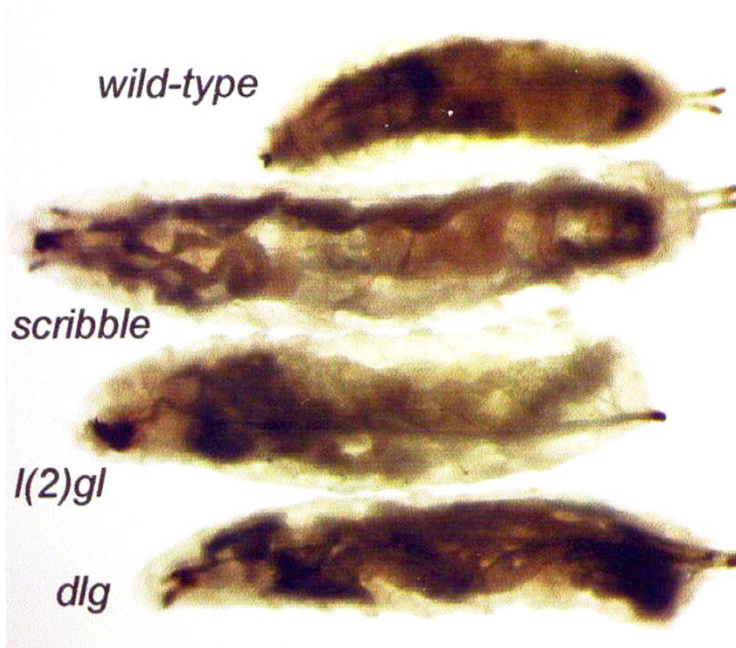
Figure 1. Size comparison between wild type 3rd instar larvae and *scrib, l(2)gl* and *dlg* giant larvae.

2. *Drosophila* as a unique model system to study tumor suppression

In order for an animal species to serve as a model of human biology it must fulfill two key criteria. The model system should be amenable to a broad set of experimental approaches and to be similar enough to humans so that findings from the model system can be exported to higher organisms and facilitate research in humans. *Drosophila*, being at the forefront of genetic research for the past one hundred years, together with the high degree of conservation with humans at the gene and cellular level, has proved itself as an essential partner in discoveries related to genetics, cell biology, human disease and cancer metastasis.

There are also many technical advantages in using *Drosophila* over vertebrate models. Flies are easy and inexpensive to culture in laboratory conditions, have a much shorter life cycle, produce large number of offsprings with feature-rich morphology and large numbers of externally laid embryos that can be genetically modified in numerous ways. Each female fly lay up to approximately 50 to 100 eggs per day for up to 20 days. It takes approximately 10 days at 25°C for an embryo to develop into a fertile adult fly. Thus, it is easy to generate large numbers of embryos or flies for experimental studies and genome-wide screens. Moreover, there are generally few limited restrictions on their use in the laboratory as there are minimal ethical and safety issues [31, 32].

2.1. Drosophila in a century of "tool-building" research

The first documented use of *Drosophila* in the laboratory was in William Castle's group at Harvard in 1901, but the "father" of *Drosophila* research is without doubt Thomas Hunt Morgan [33]. It was almost 100 years ago that Thomas Hunt Morgan reported the identification of the *white* gene in *Drosophila* [34]. Morgan greatly refined the theory of inheritance first proposed by Gregor Mendel, by using *Drosophila* to define genes and establish that they are found within chromosomes, long before it was even established that the DNA is the genetic material [31, 35]. Morgan won the Nobel Prize in Physiology or Medicine in 1933 "for his discoveries concerning the role played by the chromosome in heredity". One of Morgan's students, Hermann Muller, won the Nobel Prize in Physiology or Medicine in 1945 "for the discovery of the production of mutations by means of x-ray irradiation". Using *Drosophila* in 1920s, Muller discovered that x-rays cause massive increase in the rate of mutations and that the mutations can be passed from one generation to the other [36]. The genetic approaches used in the first 50 years of research in *Drosophila* (1910-1960), led to the development of important concepts and tools e.g. balancer chromosomes, that allowed the study of many other biological processes in the years to come [35, 37].

Interestingly, researches realized in the early fifties that genetic approaches could be used to study problems other than heredity. The continuous development of research tools between the years 1960-2010 has driven numerous new discoveries in fruit flies. In the mid-seventies, the available genetic tools in *Drosophila* offered the opportunity to address how embryonic pattern formation is controlled and to identify the genes involved in these processes [37, 38]. By carrying out a systematic chemical mutagenesis screen on the different fly chromosomes and analyzing the larval cuticle patterns, Nüsslein-Volhard and Wieschaus identified 139 genes that affect the development of the fly larva [39-41]. This analysis led to the identification of numerous genes participating in the Hedgehog, Wingless, Decapentaplegic (the *Drosophila* Tumour growth factor-β; TGF-β), and Notch pathways. In 1995, Christiane Nüsslein-Volhard, Eric Wieschaus and Ed Lewis won the Nobel Prize in Physiology or Medicine "for their discoveries concerning the control of early embryonic development". These findings have clearly demonstrated the power of forward genetics in solving complex developmental questions. Further genetic screens shed light on factors involved in various developmental aspects such as neuronal migration and growth cone guidance, circadian rhythms, learning and memory [37]. All these studies proved that despite the great morphological differences between flies and humans, many of the underlying building blocks and cellular processes are strikingly similar and conserved through evolution [31].

The range of genetic tools that have become available for *Drosophila* over the past century surpass by far those of any other multicellular organism [31]. Two experimental key features, namely the successful and efficient removal or addition of single genes or gene products, are important for any model organism to be successfully used in the laboratory. In *Drosophila*, genes can be inactivated in a random fashion using chemical mutagenesis followed by screening for specific phenotypes. Current tools allow very rapid mapping of chemically induced mutations that have robust phenotypes, permitting the isolation of null alleles, hypomorphs, neomorphs as well as conditional alleles, making possible the functional analysis

of single genes. These screens, combined with duplications and deletions covering almost all chromosomes, have greatly facilitated gene mapping. The recent improvements in whole genome sequencing techniques and single-cell profiling will enhance even more the speed of accessibility to genomic information [37, 42]. Apart from the imprecise excision of transposable elements [43], one can create mutations by selective removal or replacement of sequences using the "targeted-knockout" technology [44], as well as by using RNA interference (RNAi) to reduce expression on any gene in a tightly regulated temporal and spatial pattern [45, 46].

The use of P-element-mediated transformation, available since 1982 [47] has allowed the insertion of single genes and any DNA fragment of interest in the fly genome, and has opened the field to even more sophisticated genetic manipulations [37]. This technique was significantly improved with the P[acman] technology that allows the insertion DNA fragments in specific docking sites spread throughout the *Drosophila* genome [48, 49]. Efficient transformation has been achieved by using the Flipase-Flipase Recognition Target (FLP-FRT) recombination system, which enables the creation of mutant patches of tissues or cells in an otherwise heterozygous background [50, 51]. This system led to the development of a highly efficient "mitotic recombination system" that knockouts defined genetic functions in specific cells, tissues and organs. The yeast site-specific recombinase FLP, coupled with centromere-linked insertions of the FRT target site on all major chromosome arms of *Drosophila* [52], allows the generation of genetic mosaics, within an otherwise wild type organism, by removing almost every fly gene function. Generation of genetic mosaics is particularly useful for elucidating the function of genes which, when mutated, would otherwise kill the organism and subsequently laid the carpet in understanding how cells within tumors can interact with their surrounding microenvironment. A considerable improvement of the FLP-FRT system for analyzing mosaic tissue was the development of the MARCM (Mosaic Analysis with Repressible Cell Marker) technique [53]. Prior to the introduction of MARCM, homozygous mutant cells were identified by the absence of a visible marker such as GFP or *lacZ* in comparison to the surrounding heterozygous environment and the wild type "twin clone". By using the MARCM technique the homozygous clones can be positively marked using e.g. GFP or RFP, which can be of particular importance for the analysis of single cells in a disease model.

Another use of the P-element-mediated transformation facilitated the development of the *UAS-GAL4* system in order to ectopically express or overexpress a gene of interest in almost any tissue or cell [54]. The binary *UAS-GAL4* system allows a gene DNA sequence fused to the UAS (upstream activating sequence) to be ectopically expressed via the enhancer/ promoter of a second gene that drives synthesis of the UAS-binding GAL4 transcription factor. Thousands of *UAS* and *GAL4* fly lines are now available and their use can either modify or even abrogate gene expression in selected cell populations, in a specific temporal and spatial pattern [37, 55, 56]. Moreover, the "*Vienna Drosophila RNAi* Center" and "Bloomington Stock Center" house a collection of transgenic fly lines carrying inducible *UAS-RNAi* constructs against single protein coding genes. Currently they accommodate over 22.000 different transgenic lines, which provide knockdowns for over 90% of *Drosophila* genes. Further development of the *UAS-GAL4* system led to the TARGET (temporal and regional gene expression targeting) system, which utilizes a temperature-sensitive form of GAL80 repressor that binds GAL4 and

blocks its transcription activity at the restrictive temperature, while a shift to a permissive temperature results in GAL80 losing the ability to bind GAL4 [57, 58].

Finally, P-element technology also allowed the tagging of most genes *in vivo* e.g. with GFP, RFP or YFP, permitting sophisticated manipulations in a genomic context [59]. Transgenic flies containing enhancer-trap or protein-trap versions of individual genes allowing the analysis of the gene expression pattern and protein localization are available at "FlyTrap" (http://flytrap.med.yale.edu/index.html) [60-63] and "FlyPROT"   (http://www.flyprot.org/index.php) [64].

2.2. *Drosophila* is a model system relevant to human biology

While *Drosophila* has long served as a model for basic biological research, more recently its potential as a model for unraveling molecular mechanisms of human diseases has become widely appreciated, and numerous publications and conferences illustrate the use of *Drosophila* to unravel the mechanisms of human diseases [65-69]. Release of the first sequence of the *Drosophila* genome in March 2000 (11 months ahead of the human genome release) allowed the actual comparison to the human genome. This comparison has greatly consolidated *Drosophila's* legitimacy as a model organism for medical research [31, 70]. The sequence and annotation are freely available in "Flybase", an outstanding online database combining all current knowledge on single *Drosophila* genes including sequence and expression data, mutations, interactions and up to date scientific references. Comparative studies have shown that the molecular mechanisms underlying the development of *Drosophila* and humans are highly conserved. Since the *Drosophila* genome contains functional homologues of nearly 75% of the human disease genes, we can understand why this aspect of *Drosophila* research continuously expands. Moreover, over 85% of human genes that have been associated with a disease, have a *Drosophila* counterpart. These findings constitute a strong basis for the continuous expansion of *Drosophila* research in relation to human diseases.

What makes *Drosophila* also practical in the analysis of gene function is the nearly complete absence of genetic redundancy. The duplication of the vertebrate genome during evolution has resulted in the occurrence of multiple paralogs, e.g. Hox genes that control the body plan along the anterior-posterior axis [71], with their subsequent evolution that has generated gene expansion and diversity in protein function [72, 73]. Genetic expansion means that when knocking down a gene, other genes or homologues can compensate for its function. Yet, the extra genes rarely represent novel functions as they simply allow more complex and subtle regulation of core molecular mechanisms. In this respect, the absence of gene duplication in *Drosophila* provides the advantage of elucidating more readily the fundamental function of single genes, e.g. in tumor development, and then apply it back to vertebrates and humans as the mechanism is very likely conserved [74]. Indeed, lack of redundancy can expose the physiologically relevant phenotype of gene homologues.

The similarities between flies and humans are further supported by the fact that components of signal transduction pathways and the molecular mechanisms involved in specification, development, cell cycle regulation and human diseases were first identified in flies. The genes, which have been characterized in flies, were subsequently studied in mice and humans, and

their names were adopted or adapted from their *Drosophila* homologues. For example, the mammalian Notch 1-4 named after the *Drosophila* Notch (fly wings having a large notch on the wing), "*sonic hedgehog*" named after the *Drosophila* "*hedgehog*" (round larvae with extra bristles) and *Wnt* from the *Drosophila* "*wingless* and *INT*-related" gene [31].

3. Recent advances in modeling tumor progression and metastasis in *Drosophila melanogaster*

Since the discovery of oncogenes and tumor suppressor genes, intense research in many laboratories all over the world has brought us to the point where we are starting to understand the main principles underlying molecular changes in the course of tumor progression [3, 75]. The development of new technologies revealed the complex molecular nature of tumorigenesis in which tumor progression can be envisaged as a network of simultaneous events within both tumor cells and the stroma. Because cancer is an age-associated disease in humans, using *Drosophila* to model cancer development, progression and metastasis was debatable [76, 77]. However, over the last decades the study of *Drosophila* has significantly contributed to the understanding of key cancer events, including the loss of cell polarity, the competition between tumor and normal cells, as well as metastasis [78].

In addition to the importance of tumor cells themselves, their neighboring cells and the surrounding stroma are now recognized as important regulators of cancer progression [79]. Cell competition is a type of short-range cell-cell interaction in which cells expressing different levels of a particular protein are able to discriminate between their relative levels so that the one cell, the "loser", disappears from the tissue whereas the other, the "winner", survives and proliferates to cover the space left by the disappearing cell. Some tumor-promoting mutations in *Drosophila* are able to induce cell competition between the cancer cells and their micro- and macroenvironment [80-86]. Metastasis is the latest phase of cancer progression during which cells detach from their original niche to invade distant tissues [87]. For several decades, our understanding of the molecular processes leading to metastasis was largely derived from studies of cancer cell lines *in vitro*, xenografts of human tumors and a limited number of transgenic or knockout mouse models [1, 88, 89]. However, understanding the individual steps leading to tumorigenesis or analyzing multiple genetic interactions in mice is difficult. Existing models are currently being re-evaluated given the increasing awareness of tumor complexity. Therefore, using a model system that allows the efficient analysis of the combinations of genetic events that trigger tumor initiation and metastasis during cancer development is the major challenge in cancer research at the moment. *Drosophila melanogaster* provides a model of choice for cancer analysis as the collection of sophisticated genetic manipulation techniques have been invaluable for dissecting signaling pathways that affect cell specification, differentiation and growth [90-93]. Indeed, *Drosophila* cancer models are very helpful in unraveling the chrono-logical sequence of events leading to human cancer. For example, in metastasized human tumors elucidating the identity of the initial mutations is often tedious as oncologists are in most cases looking at the end point of the disease progression. Finally, *Drosophila* genetics is

very powerful as we can dissect the triggering events initiating cancer and the subsequent steps leading to the progression of the disease [94].

3.1. Modeling cell competition and metastasis

With these added complexities in mind, the analysis of cancer-disposing mutations in only a subset of cells or in clones within the context of a wild type surrounding tissue is gaining more interest because it offers a reasonable approximation of the clonal nature of human cancers, compared to the analysis of the multi-step model of tumor progression in the context of a whole organism. A great number of very interesting publications provided us with information about new and unexpected findings on the role of the polarity genes *scrib*, *lgl* and *dlg* in cancer initiation, progression and metastasis. Nowadays, it becomes obvious that they play a broader role than initially thought, through the cooperation with individual partners and signaling pathways and have helped us to understand the role of cell competition and of the tumor microenvironment during tumor survival and progression.

Analysis of *scrib*⁻ mutant clones in the *Drosophila* eye imaginal discs has shown that tumor development is suppressed by the JNK-mediated apoptotic pathway activated by the surrounding wild type cells, whereas the neoplastic and metastatic potential is regained through the synergistic effect of a simultaneous up-regulation of Ras signalling within the same clones [84, 95, 96]. These results underline the effect of the surrounding normal cells on the transformed *scrib*⁻ clonal cells, which leads to a cell competition similar to the one observed in the mammalian cancers [1, 96-100]. In a model for *scrib* tumorigenesis, the analysis of the downstream pathways in *scrib*⁻ epithelial clones revealed that excessive cell proliferation is restrained by JNK-mediated apoptosis. Upon simultaneous activation of either Ras or Notch, JNK-mediated apoptosis is blocked, and Ras/Notch together with JNK cooperatively promote tumor growth and invasion [96]. In other words, whereas JNK activity normally promotes the apoptosis of *scrib*-deficient cells, it becomes a driver of cellular overgrowth, tumorigenesis and invasion in the presence of oncogenically activated Ras or Notch signalling [76, 101]. These tumors present similar characteristics to human cancers that lack Scrib, including basement membrane and extracellular matrix degradation, loss of E-cad expression, combined with migration, invasion and secondary tumor formation [101] Another report provided a molecular link between loss of polarity and tumorigenesis, since *scrib*⁻, *dlg*⁻ and *lgl*⁻ clonal cells in a wild type surrounding become metastatic only in combination with *Ras*^{v12} activation, resulting in JNK activation and E-cad inactivation [102]. The analysis of the JNK-mediated tumorigenesis, which in *Drosophila* cells reveals a cooperation with Ras similar to that taking place in mammalian breast epithelial cells, indicates that the knowledge gained from the analysis in *Drosophila* can help us elucidate tumor formation in the mammalian system.

Mutations inactivating the Salvator-Warts-Hippo (Sav-Wts-Hpo) pathway can also cause super-competition, contributing to the overgrowth of cells expressing these mutations in the presence of wild type cells [80, 103]. Since the first discovery of the Sav-Wts-Hpo pathway in *Drosophila*, the role of these genes in restricting cell growth and proliferation, and inducing apoptosis has triggered a great interest in its study. The components of this pathway act as important tumor suppressors that regulate tissue growth by promoting cell cycle exit and

apoptosis [80, 104-112]. Recent data from mammals and *Drosophila* show the occurrence of a very conserved pathway that links the pathway of cell polarity to the regulation of tissue growth.

In the model of Scrib tumorigenesis, induction of apoptosis in *scrib* clones could not explain how *scrib*- cells are prevented from overproliferating. This was answered by the finding that cell competition between *scrib* and wild type cells prevents overproliferation by suppressing Yorkie (Yki; a transcription factor, which is suppressed by the Sav/Wts/Hpo pathway) activity in *scrib*- cells [113, 114]. Suppressing Yki activation is critical for *scrib*- clone elimination by cell competition. Cell competition leads to activation of JNK in *scrib*- cells and JNK antagonises Yki activity, which leads to elimination of the clone. Experimental Yki elevation is sufficient to promote neoplastic growth in *scrib*- cells [114]. Along the same line, when *lgl* is mutated in a mosaic tissue, the *lgl*- clonal cells become the "losers" in cell competition. However, simultaneous overexpression of the Ras signalling pathway or of *yki* in *lgl*- clones, causes overgrowths and JNK-mediated apoptosis at the periphery of the transformed clones [115-120]. Moreover, JNK-mediated elimination of *lgl*- clonal cells is relieved and the overgrowth potential is re-established by upregulation of c-Myc, demonstrating the the death of *lgl*- clones is essentially driven by c-Myc-induced cell competition [121]. Simultaneous downregulation of the *lgl* and the JNK pathway in the whole-animal system results in a phenotypic reversion of tumor growth, absence of the giant larvae and recurrence of pupariation, thereby showing that JNK activity is essential for overgrowth and invasion of *lgl* tumorous discs [122]. Moreover, in the developing *Drosophila* eye and imaginal disc epithelia Lgl, αPKC and Crumbs proteins regulate cell proliferation and survival by controlling the activity of the Sav-Wts-Hpo pathway [115, 123-125].

Among the wide palette of cellular events leading to JNK activation is the dTNF (tumor necrosis factor)/Eiger. Eiger is the only *Drosophila* member of the TNF superfamily and its deregulated expression in imaginal disc cells results in JNK-mediated apoptosis [76, 126]. JNK-dependent cell death in *scrib* and *dlg* clones requires dTNF, acting as a "tumor death factor" [127]. On the other hand, in tumors deficient for *scrib* and *dlg* that also express *Ras*, the TNF signal is converted into a signal, which promotes tumor growth and invasion [126]. More precisely, upon dTNF downregulation, cell death in *dlg* and *scrib* clones was blocked and *in situ* outgrowths appeared, probably by TNF-mediated extra-cellular matrix (ECM) remodelling [76, 126]. When generated in an *eiger*- mutant background, $Ras^{v12}scrib$- clones displayed non-invasive *in situ* overgrowth. Similarly, in whole $Ras^{v12}scrib$-$dTNF$- animals, development proceeded up to pupal stages, overcoming the "giant larvae" phenotype [76, 126]. These recent results suggest that several of the critical overgrowth phenotypes of *scrib*, *dlg* and *lgl* in the clonal and whole-tissue context are mediated by dTNF and that dTNF pro-tumor function depends partially on JNK activation in tumor cells, which provides a switch from *in situ* to invasive growth. Immunostaining experiments detected dTNF in a punctuated, intracellular vesicle pattern at the periphery of hemocytes in association with the *dlg*- clones, indicating that dTNF expression in hemocytes is sufficient for dTNF/JNK pathway activation within the *dlg*- clones, and mark the importance of hemolymph and non-cell autonomous immune response in tumor progression [76, 126].

Until very recently, the mechanism by which surrounding normal tissue exerts antitumor effects against *dlg*, *scrib* or *lgl* clones remained elucive. New results from clonal analysis in *Drosophila* imaginal discs have shown that JNK activation from the wild type surrounding leads to upregulation of PVR, the *Drosophila* PDGF/VEGF receptor, that activates the ELMO/Mbc phagocytic pathway, which in turn eliminates the oncogenic clonal cells by engulfment [128]. From an evolutionary point of view, the development of such mechanism, which senses and eliminates "neoplastic" tumor-suppressor mutant cells such as those of *scrib*- and *dlg*- but not "hyperplastic" ones (in which despite of overproliferation, cells are normally shaped and retain a differentiated epithelial monolayer, such as those of the Hippo pathway and PTEN) [128], shows the necessity to specifically eliminate the high-risk malignant neoplastic cells before they confer any harm to the organism. Taken together, these studies demonstrate that hemocytes together with the tumor microenvironment act as regulators of epithelial delamination required for tumor invasion. Due to the ease of genetic manipulations, *Drosophila* research can bring meaningful insights to our understanding of the mechanisms of communication between cancerous and normal cells as well as between tumor tissue and the immune system [87].

3.2. *Drosophila* provides critical insights on how conserved mechanisms contribute in cancer and tumorous development

Drosophila research has also contributed to cancer analysis by identifying genes and signaling pathways later found to be critical for tumorigenesis in mammalian systems. In several cases *Drosophila* has been used to establish specific model systems in order to understand processes that seem to be more complex in vertebrates and mammals [81, 101, 129, 130]. One of the most extensively studied *Drosophila* models of tumor biology is the asymmetric cell division of neuroblasts, the *Drosophila* neuronal stem cells. In *Drosophila* neuroblasts (NB), the PAR3-PAR6-atypical protein kinase C (aPKC) complex segregates apically and recruits the adaptor protein Inscutable (Insc), which connects this complex to the partner of Insc (Pins), guanine nucleotide associated protein-α_1 (Gα_1), mushroom body defect (MUD), and p150^{glued} to the crescent directing the orientation of the mitotic spindle during asymmetric cell divisions. aPKC promotes the exclusion of partner of numb (PON), Lgl and Numb, which along with Miranda (Mira), Brain Tumor (Brat) and Prospero (Pros), localize to the basal crescents [101, 130, 131]. Interestingly, most of these genes have functional mammalian homologues and a very recent study points out the role of the Par3 in mammalian skin tumorigenesis [132]. In mouse skin tumorigenesis, Par3 deficiency results in reduced papilloma formation and growth. Further-more, Par3 expression is reduced in both mouse and human keratoacanthomas, indicating the tumor-suppressive properties of Par3. More insights into tumor physiology came from a very interesting study in *Drosophila* NBs that indicates the critical role of starvation in promoting the overgrowth of *pins* larval brains. Energy stress, mediated by loss of TOR and Phosphati-dylinositol 3-kinase (PI3K) components, in combination with loss of *pins* results in loss of asymmetric NB division and brain overgrowth (Rossi, 2012). Since the PI3K and TOR signaling pathways are vital to the growth and survival of mammalian cancers [133, 134], using *Drosophila* in order to dissect the cross talk of these pathways to preexisting tumor suscepti-bility defects e.g. polarity defects, in a simple animal model is of great importance.

The usefulness of *Drosophila* is further illustrated in the development of a *Drosophila* model for human brain cancer. Glioblastoma (GMB) is the most frequent and malignant form of high-grade glioma that infiltrate the brain, grow rapidly and are refractory to current therapies [135]. One key to development of new and effective therapies against these tumors is to understand the fundamental genetic, cellular and molecular logic underlying gliomagenesis. Signature genetic lesions in glioblastomas include mutation of the epidermal growth factor receptor tyrosine kinase (EGFR) and mutations activating components of the PI3K pathway. *Drosophila* studies using lineage analysis combined with cell-type specific markers demonstrated that EGFR-Ras and PI3K can induce fly glial neoplasia through activation of a combinatorial genetic network composed in part of other genetic pathways also commonly mutated in human glioblastomas [135]. In the future, large-scale forward genetic screens with this model may reveal new insights into the origins of glioblastoma and may also provide new therapeutic strategy for the treatment of this form of human tumor.

Drosophila has been also used to elucidate the molecular mechanisms of human hereditary diffuse gastric cancer (HDGC) [136]. Gastric cancer, as several human cancers, originates from epithelial cells. Mutations in the CDH1 gene, which encodes the cell adhesion molecule E-cadherin (E-cad), are associated with HDGC in humans. In order to understand the role of E-cad in this disease, a *Drosophila* model has been developed in which mutated forms of E-cad can be studied *in vivo* [136, 137]. Moreover, genetic and molecular studies of *Drosophila* hematopoiesis can also contribute to our knowledge of the hematopoietic niche and hematopoietic malignancies in humans. Vertebrate hematopoietic stem cells give rise to an hierarchically organized set of progenitors and deregulation of the hematopoietic differentiation program can lead to numerous pathologies including leukemias. With the discovery that many transcriptional regulators and signaling pathways controlling blood development are conserved between humans and flies, *Drosophila* is particularly suitable model for investigating the mechanisms underlying the generation of blood cell lineages and blood cell homeostasis [138].

Interesting results using a *Drosophila* cancer model demonstrated that apoptosis activation in differentiation-compromised cells constitutes a mechanism for early prevention of cancer [139]. Apoptosis is a highly conserved cellular function to remove excessive or unstable cells in diverse developmental processes and disease-responses. An important example is the elimination of cells unable to differentiate, which have the potential to generate tumors. Using cell-type specification in *Drosophila*, Ingrid Lohmann and her colleagues identified a conserved regulatory mechanism that underlies cell-type specific removal of uncommitted cells by apoptosis as a cancer prevention mechanism [139]. Under normal conditions the transcription factor Cut activates differentiation, while it simultaneously represses cell death via the direct regulation of a pro-apoptotic gene. However, loss of Cut and subsequent release of apoptosis leads to overproliferation of the mispecified cells that can acquire metastatic potential in a sensitized background. Importantly, this regulatory wiring is also found in vertebrates in which other cell-type specification factors may similarly be employed to suppress tumor formation. Thus, coupling of differentiation and apoptosis by individual transcription factors

1	*Drosophila* flies are easy and inexpensive to culture in laboratory conditions, have relatively low set-up and maintenance costs
2	Short life cycle
3	High fecundity (produce large number of off-springs with feature-rich morphology)
4	The *Drosophila* community is open and generous in sharing reagents within the community.
5	No ethical issues and regulatory considerations.
6	Genetic advantages • flies have only 4 pairs of chromosomes • males lack genetic recombination, making genetic crosses easier • flies tend to lack genetic redundancy
7	Signaling pathways controlling growth, differentiation and development, which are involved in tumor formation in the fly are largely conserved between *Drosophila* and humans
8	Availability of numerous genetic tools & reagents for generating mutants and analysis of gene expression by using methods producing over- & ectopic- expression. • The use of "balancer chromosomes" with multiple DNA inversions prevent female recombination and allows the perdurability of mutations on the original carrier chromosome • Wide variety of gene targeting strategies, e.g. UAS-GAL4 system combined with RNAi knock-down allow the tissue-specific analysis of tumor suppressor gene and oncogene function • Mosaic analysis of animals containing mutant clones next to wild type tissue, using FLP-FRT and MARCM recombination systems, allows the analysis of tumor microenvironment in invasion, metastasis & inflammation.
9	Possibility to perform genome-wide screens using chemical mutagenesis, tissue-specific RNAi knockdown, effectors and modifier screens to identify genes involved in specific developmental pathways and assign and validate new gene functions.
10	Use of *Drosophila* tumor models for pharmacological screening and development of new therapeutic strategies for cancer.

Table 1. Advantages of *Drosophila melanogaster* for the analysis of cancer.

is a widely used and evolutionarily conserved cancer prevention module, which is hard-wired into the developmental program [139].

Furthermore, genetic analysis of border cell migration in the *Drosophila melanogaster* ovary provides clues that improve our understanding of ovarian cancer metastasis at the molecular level that might also lead to therapeutic targets [140]. The border cells of the *Drosophila* ovary provide a particularly simple example of cell migration in which cells derived from an epithelium invade a neighboring tissue. The large numbers of genes that control border cell migration identified in genetic screens emphasized also the requirement of multiple extracellular signals for border cell motility [141]. Interestingly, the motile and invasive properties of the border cells seem to share common characteristics with mammalian ovarian carcinoma cells. Based on work done in *Drosophila*, the function of some mammalian proteins such as myosin VI, have been tested for their ability to regulate motility of ovarian carcinoma cells.

Another example can be found in the EGFR pathway, which functions redundantly to PVR to stimulate border cell migration [142]. Overexpression of EGFR has been reported in up to 70% of ovarian carcinomas [143]. The role of E-cad is also here critical. Normal cells of ovarian surface epithelium express little or no E-cad. However, many primary ovarian carcinomas, similar to border cells, express E-cad at the cell surface and in the cytoplasm [144, 145]. Although cell-surface expression of E-cad is reduced in many metastatic carcinomas, these tumors frequently still have detectable intracellular E-cad [146], indicating that these cells, similar to border cells, have acquired the ability to downregulate E-cad activity at a post-transcriptional level [140].

Numerous molecules identified in *Drosophila* genetic screens have proven to be important to human cancers [140, 147]. For example, the Hedgehog gene and the Wnt homologue Wingless were originally identified in genetic screens for mutations that disrupt embryonic patterning. Subsequent studies of signaling proteins such as Hh, Wnt [140, 148-150], Notch [151, 152] and RhoGTPases [153] as well as of integrin-related adaptor proteins [154, 155] and Hox genes [156, 157], revealed crucial functions not only in normal mammalian development but also in various cancers. It is therefore well accepted that genetic approaches to the study of normal cellular behavior in simple model organisms can yield fundamental insights into the molecular underpinnings of cancer.

4. New perspectives in modeling tumorigenesis in *Drosophila melanogaster*

Drosophila is emerging as a valuable system for use in clinical drug discovery and therapeutic process [52, 129, 158-160]. So far, *Drosophila* was not a favorable model in drug discovery. The main reason was that "Drosophilists" were mainly concerned with answering fundamental development and cell biology questions, and elucidating principles of basic mechanisms and not practical issues of therapeutics [129]. This view is slowly changing as interest in therapeutics and pressure for practical outcomes increases, and combined with the development of powerful tools allows *Drosophila* to catch-up in the field.

The remarkable degree in conservation of biochemical pathways that control processes such as cell proliferation, differentiation and migration as well as nervous system function in behavior and cognition, sustains perfectly the use of invertebrate model genetic organisms as tools for drug discovery and validation [52]. *Drosophila's* genetic and genomic tools can be adapted to build sophisticated disease models for studying cancer and metastasis, and for therapeutic development. While testing with mammalian models is essential prior to approval for human trials, the use of invertebrate animal models that are amenable to molecular genetic manipulations, provides experimental and biological advantages that can streamline drug discovery and testing process. Among the benefits of a genetics-based approach is the ability to screen for proteins that may be novel drug targets, and in genetic backgrounds that could more accurately reflect a specific disease state [52]. New drugs can be tested in *Drosophila* much

faster than in mammalian models and can even be used for high throughput screening processes as an alternative to cell culture [31].

Drosophila may constitute an appropriate model system that can be used for screening a "whole animal setting" as an alternative to cell-cultured based methods. In most pharmaceutical industries the discovery of new compounds with potential positive pharmacological effect relies on the screening of small molecule libraries for interactions with purified proteins, or for the ability to induce a desired physiological response in cultured cells [52]. One of the main problems is that complex processes such as tumor metastasis cannot be recapitulated in a cell or an organ culture. Moreover, cells and organs are physiologically connected and their interplay could be critical in the development of some diseases. Furthermore, the time component of the disease progression cannot be easily recapitulated *in vitro* [161]. The second problem is that after the initial screen, the next phase of drug testing, which requires the use of intact mammalian animal models to assay the effectiveness of candidate compounds, usually fails. If in this step of drug discovery process, the compounds isolated *in vitro* or in cell culture, are invalid in mouse, the result is an enormous waste of funds and efforts [158]. At the same time, whole animal vertebrate models are not particularly suitable for high-through-put methodologies and if then only at an extremely high cost. To by pass these limitations, efforts are now being made to screen chemical libraries on whole-animals like *Drosophila* with genetic amenability, low cost and culture conditions compatible with large-scale high-throughput screening.

Furthermore, performing drug screening in the *Drosophila* "whole animal system" does not dependent on the prior identification of a target and permits the selection of compounds with an improved safety profile. A screen based on a phenotypic observation has the advantage of being independent of the specific molecular target involved. Then, depending on the readout used to assay the effectiveness of the candidate drug compound, a large variety of bio-active molecules may be detected in the same screen [158]. Finally, similar to the established "en-hancer" or "suppressor" screens, *Drosophila* gives the possibility to test chemical libraries in the genetic background of a disease for their ability to reverse the abnormal phenotype to wild type or partially rescue the disease phenotype [158, 161].

Several groups today develop the associated technology to use *Drosophila* in the first phase of screening for drug compounds, and subsequently test them in more expensive mammalian models. Moreover, the fact that it is usually easier and more straightforward to manipulate the genetic background of *Drosophila* and mimic a disease state, opens also new possibilities for efficient drug testing in a disease-related content. For example, the development of genetically modified animals with fluorescently tagged proteins would allow the use of standard plate-reader spectrofluorometer for whole-animal screens [161].

When the development of mosaic tissues is essential for the analyses of a disease model, the use of MARCM provides notable advantages for effective drug discovery. One is the ability to follow the morphology of mutant cells and tissues which could be useful for assessing the efficacy of a therapeutic compound [52]. When a mutation in a given gene causally produces a disease, it is possible that this mutation elicits a change in expression of other genes and in the function of proteins. These alterations may contribute to the pathologies associated with

the disease. The characterization of these changes constitutes then the first step needed to develop rational therapeutic strategies. Finally, the MARCM methodology should provide ways to identify mutant cells or tissues for a given gene, isolate and subject them to proteomic and genomic analyses which would determine modifications in gene expression and protein interaction profiles [52].

The phenotypes of complex trait diseases such as obesity, heart disease and cancer are the result of modifications occurring in multiple biochemical pathways. The disease phenotype can be caused by improper activation or inhibition of a protein that acts in any of the contributing pathways. Restoration of the normal phenotype would be expected if the output from the primary biochemical pathway affected is rescued via drug-based therapy [52]. However, if multiple pathways contribute to a phenotype, it stands to reason that modifying the activity of a parallel pathway could also elicit a positive therapeutic effect. The use of genetic model organisms has long been a successful means for elucidation of biochemical and physiological pathways, and one of the most powerful strategies available for uncovering genes that act together in producing a phenotype is a search for genetic interaction or a modifier screen. Modifier screens work by generating animals with a mutation in a gene of interest that elicits a sensitized phenotype, and then screening for mutations in progeny that enhance or suppress (i.e., modify) the primary phenotype [52]. *Drosophila* disease models are currently used in drug screening for treatment of diseases such as Alzheimer's and Huntingtons's disease, Fragile X Syndrome and muscular dystrophy [160, 161]. The use of drug discovery especially for muscle diseases is of particular importance as the muscles are difficult to reconstitute *in vitro* and elucidation of the physiology of muscle related diseases and relevant treatment is still poorly understood. However, the similarity in architecture, composition and function between *Drosophila* and vertebrates may trigger studies in the fruit fly and provide these diseases with some valuable therapeutic answers [161].

Drug discovery has also proved very effective for the identification of cancer treatments such as the multiple endocrine neoplasia type-2 (MEN2) [129, 162]. MEN2 is a "one-hit" solid tumor syndrome, characterized by mutations in the Ret protein, a transmembrane receptor tyrosine kinase. Patients with mutated oncogenic isoforms of Ret, develop medullary thyroid carcinomas (MTC) that lead to metastasis, which seem to be resistant to traditional chemotherapies. To develop a whole-animal transgenic model, various oncogenic Ret isoforms were targeted to the developing fly eye epithelium. The fly "rough-eye" phenotype is characterized by eye overgrowth, switch in cell fate and other aspects, proving the effectiveness of fly as model and useful readout for screening. The screening for clinical relevant compounds, in the tumorous developing flies by Cagan and his group resulted in the identification of Vandetanib, a broad kinase inhibitor, which Cagan called "the worst kinase inhibitor". Although not very specific and effective, this kinase inhibitor was indeed effective in rescuing the fly phenotype because it regulated the activity of other kinases such as Ras, Src and PI3K (all of which are involved in cancer). Other compounds were more capable of rescuing the rough eye phenotype but reduced animal viability. Yet, Vandetanib displayed little toxicity to the animal as a whole, indicating that tumors might display a lower tolerance threshold for drugs than the entire animal. Obviously the "off-target" effects of Vandetanib, by suppressing kinases other than

Ret, are important for its effectiveness. Classical drug screenings would reject Vandetanib as too inefficient to the target and too low in its specificity [129, 162]. Cell-culture and subsequent fly work proved to be extremely valuable, as Vandetanib was shown to be efficient in Phase II clinical trials. Currently Vandetanib is in Phase III of clinical assays. This further proves the power of *Drosophila* not only for clinical relevant drug discovery but also for shaping how we should approach drug discovery for treating diseases.

A new *in vivo* drug screening in *Drosophila* has been performed to target cancer stem cells (CSCs) in the group of Norbert Perrimon [163]. Cancer cells represent a small number of cells within tumors with a self-renewing capacity that can regenerate tumor cells types through their stem cell-like renewal capacity. Their resistance to chemotherapy is the main reason why chemotherapeutic treatments are ineffective and the disease often relapses. In order to cope with the challenge of finding drugs that specifically target the CSCs, the Perrimon laboratory uses the *Drosophila* gut as the stem cell system to develop novel methods and approaches to screen for anti-cancer drugs that target CSCs *in vivo* in the gut microenvironment. By directing the expression of oncogenes in *Drosophila* transgenic fly models combined with a screening method that involves monitoring of tumor size by luciferase reporter activity, they identified 25 compounds that reduced tumor size. Further confirmation was validated with dissection of the gut, histochemical-imaging of the gut specific cells and determination of the specificity of the drugs. For example, some drugs were targeting only the CSCs whereas others were targeting CSCs but at the same time promoted growth of the wild type stem cells and in some cases also affected stress pathways in the daughter cells.

5. Limitations in using *Drosophila* as a model system: how far can we go?

Drosophila melanogaster, as a model system for studying tumorigenesis and human disease has certain limitations, especially in regard to the biological processes that evolved only within the vertebrate lineage [164]. The main limitation of *Drosophila* arises from the fact that some diseases cannot be modeled because the corresponding genes and organs present in humans are missing in flies [161]. For example, *Drosophila* has a single cardiac chamber that functions as a heart in the context of an open circulatory system. Moreover, the *Drosophila* myocardium receives oxygenation through diffusion and does not have coronary arteries [165]. A second limitation arises from differences in cellular and molecular processes of *Drosophila* in comparison to humans. For example, there are cases in which one or several key molecules mediating a human disease-specific pathway are missing in flies [160]. Ultimately, some areas such as learning, endocrine function and mammary gland development, may prove difficult to study in a simple invertebrate like *Drosophila* and so the study of these particular disorders may not benefit substantially from *Drosophila* genetics [166]. Another example is modeling tumor metastasis. In mammals malignant cells undergoing metastasis enter the local blood or lymph vessel before colonizing a distant tissue and forming metastatic tumors. This is very difficult to model in *Drosophila* as flies have a rudimentary hematopoietic system and a dramatic different lymphatic system compared to mammals [78]. However, one should point out that despite these differences the "master regulators" of heart, eye, kidney etc. have proved to be

remarkably conserved [162]. This means that in the case of e.g. spinal malformation, flies could be used to model bone formation *per se*, but as Notch signaling has a pivotal role in regulating this process, any knowledge obtained about interactions between components of this pathway in *Drosophila* could be relevant to processes that these genes control during spinal formation in humans [164].

Are there limitations in using *Drosophila* for treatment-relevant drug discovery? The limitations in this case result from the anatomical and molecular differences of small model organisms in comparison to humans, as this may cause the elimination of a significant fraction of the hits generated. The use of *Drosophila* models should be viewed as complementary alternatives to cellular assays and *in vitro* screenings made in mammalian cells, rather than the absolute shortcut to screen drugs for human treatments [158]. Their added value for drug discovery varies from disease to disease, and mainly depends on the availability of other options. Indeed, assays in *Drosophila* are complementary to *in vitro* and cellular systems, and in comparison to rodent model systems *Drosophila's* small size and culture conditions fulfill the requirements for large-scale screens [158]. Furthermore, another limitation also results from the dose-dependence of the drug treatment. In rodent model systems the drug dosage may differ according to the mode of penetration and the nature of the drug. In *Drosophila* dose-response experiments are easily feasible but the compounds are essentially delivered to the fruit flies through the media [167]. Thus, it is important that the results obtained with *Drosophila* are compared with data obtained on laboratory rodents and, when possible, in humans. Furthermore, in numerous cases the results will be more qualitative than quantitative. Although the conclusions derived from *Drosophila* studies may remain too uncertain for pharmacologists, the data obtained from invertebrate-based screening could lead to important breakthrough, particularly for those diseases in which the pathophysiology is poorly understood [158, 161].

Often model systems are used to understand life and basic biological and cellular mechanisms. A better understanding of a specific human disease often comes as a consequence of the better overall understanding of biological processes [159]. Within this context, *Drosophila* is a valuable tool to categorize putative candidate genes for further downstream functional analysis in vertebrates. It can be used to dissect the likelihood that individual genes in a gene-cluster contribute to disease susceptibility, identify the relevance of a gene to a disease-pathway and get insights on gene specific functions manifested at the level of a tissue or involving cell-cell communication. Using *Drosophila* models, preliminary experiments with other model systems (such as mice) may be reduced and experiments in higher organisms can be better focused. Using *Drosophila* in a systematic approach together with other models and tools, seems promising in order to significantly reduce the turnout time from genetic results into biologically meaningful data [168]. Conclusively, although *Drosophila* will probably not always serve as a perfect model for human disease modeling, the common underlying molecular interactions and signaling pathways between flies and humans will continue to allow researchers to use *Drosophila* in order to answer existing questions, pose new hypotheses, get entry points in elucidating cancer and personalized cancer therapy, and complement studies from other model systems and vertebrate organisms [164].

6. The expanding role of *Drosophila* in cancer research: Bridging past, present and future

Undeniably the study of *Drosophila* has brought major contributions in understanding to the fundamentals of cancer and has further given strong impulses not only in basic but also in applied research. The unrivalled advantages and tools offered by *Drosophila* ensure that it will remain a premier research organism for many years to come. The advantages of *Drosophila* as a model system includes the availability of genetic tools developed in a century of "tool-building" research, its short life cycle and ability to unravel the basic function of genes in a straightforward way. The use of visible mutations and chromosome mapping coupled to currently available complex genetic databases including genomic and proteomic sequences, together with help of systematic gene disruption (RNAi libraries), microarray analysis, protein interaction maps and Flybase, lay the carpet for a renewed new age of research in *Drosophila*, and will allow scientists to address new questions on biological processes which were previously inaccessible. In turn, the new discoveries will foster new research and answer to more precise questions about signaling pathways and behavior of individual cells in cancer. The *Drosophila* research will continuously contribute to a better understanding of tumor formation and progression, and may thus improve therapies in treating cancer.

Could *Drosophila* still be a valid model for understanding tumor formation and could it still provide a lead for curing cancer? Although the fruit fly does not appear to be suited for studying vertebrate-specific tissues, such as brain and heart development or neural crest migration [37], *Drosophila* may still help to identify critical key-genes, discover new biological pathways, define new research approaches, and therefore pioneer numerous fields in the understanding of cancer, including vertebrate biology. *Drosophila* can also be used to unravel the sequence of events leading to tumor formation and to trace the initial stages of tumor formation. One of the main outcomes of the genome analysis has been the finding of a high degree of conservation among genes and importantly 75% of the genes involved in human diseases have homologues in *Drosophila*. There is also a high degree of functional conservation between the signal transduction pathways and a high degree of structural and functional conservation between cell adhesion proteins of *Drosophila* and humans showing that the fundamental biological processes have a common origin and has been relatively conserved during the 600 million years of evolutionary divergence between invertebrates and vertebrates. New emerging challenges in the study of tumorigenic inflammation, in *in vivo* screening for drug acting on cancer stem cells, in the therapeutic drug design and discovery will provide us with new insights into a "multi-target" approach for treating cancer. Finally, innovative technologies such as microarrays and nanotechnology, combined with novel methods in computation and bioinformatics, could be used in combination with genome-wide analysis in *Drosophila*, functional maps of the chromatin landscape [169-171], cis-regulatory map of the *Drosophila* genome and pattern of co-binding partners in transcription [172], as well as high-resolution of transcriptome dynamics throughout development [173, 174] to define more accurately the network of genes and pathways that would permit initial cancer cells to build expanding tumors. These new directions highlight not only the value of basic research but also

the intrinsic advantages of *Drosophila* as a model organism for studying the complexity of cancer.

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Polyphenolic Compounds Targeting p53-Family Tumor Suppressors: Current Progress and Challenges

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Additional information is available at the end of the chapter

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1. Introduction

The chemotherapeutic properties of polyphenols have recently received an increasing interest since it has been established that these compounds can modulate each step of the cancer progression process (initiation, proliferation, survival, migration, angiogenesis, and metastasis). Polyphenols are believed to be multi-targets drugs and in the present chapter we will give an overview of recent investigations concerning apoptosis induction by three major compounds, resveratrol, curcumin and epigallocatechin-3-gallate (EGCG) mainly through the regulation of the p53 tumor suppressor pathway. The potential regulation by polyphenols of p53 expression at the transcriptional and post-translational levels has been extensively described. Interestingly, polyphenolic compounds are also able to trigger apoptosis of numerous cancer cells, independently of the p53 status (wild-type, mutated or deficient). Moreover alternative mechanisms supported by recent studies highlight the role of p73, a p53 related tumor suppressor, as another key target for polyphenols. Then the molecular mechanisms involved in tumor suppressors (mainly p53 and p73) expression by polyphenols will be discussed with a specific focus on the role of oxidative stress which is believed to be a key element in polyphenols-induced cancer cells death.

2. Anticancer properties of polyphenols: Chemoprevention and chemotherapy

Polyphenols are natural compounds characterized by a structure containing at least one benzene ring substituted by at least one hydroxyl group. Beside this chemical hallmark,

phenolic products currently constitute a large and still expanding complex and heterogeneous family of molecules (more than 8000 phenolic structures currently known) with a great diversity of structure and size ranging from the low molecular weight simple phenols up to the high molecular weight tannins [1-3]. Polyphenols are also one of the largest and most widespread classes of constituents present in plant kingdom and more particularly in plant-derived foods and beverages giving them their color and taste properties. Polyphenols can be structurally divided into two main families: flavonoids and non-flavonoids. Flavonoids are especially abundant in fruits, vegetables, seeds, spices, herbs, tea, cocoa, and wine. The six major subclasses of flavonoids are anthocyanidins (e.g., cyanidin, delphinidin; primary sources: red berries, red cabbages, cherries, grapes, and onions), flavan-3-ols (e.g., catechin, epicatechin, EGCG; primary sources: tea, grapes, cocoa, apples, and red wine), flavanones (e.g., hesperitin, naringenin; primary sources: oranges, lemons, and grapefruits), flavones (e.g., apigenin, luteolin; primary sources: celery, parsley, and thyme), flavonols (e.g., kaempferol, myricetin, quercetin; primary sources: apples, beans, broccoli, and onions), and isoflavonoids (e.g., daidzein, genistein; primary sources: legumes and soy products). Phenolic acids represent a large subclass of non-flavonoid polyphenolic compounds which can be further divided into two main types: benzoic acids (e.g., gallic acid, ellagic acid, vanillic acid; primary sources: tea, red wine, berries, nuts, and herbs) and cinnamic acids (e.g., caffeic acid, chlorogenic acid; primary sources: coffee, berries, plum, and apple). Other important classes of non flavonoids with healthy properties are stilbenes, such as resveratrol (primary sources: red wine, berries and nuts) and curcuminoids such as curcumin the main component of dried turmeric and curry powder [4]. Polyphenols are considered as secondary plant metabolites and have been associated with several functions in plants such as resistance against microbial pathogens and insects, protection against DNA-damaging UV light, reproduction, nutrition and growth [3]. In parallel to their protective properties in plants, polyphenols have long been regarded as a pool of bioactive natural products with potential benefits for human health. Plant extracts, herbs and spice containing these compounds have been used for thousands of years in traditional medicines. Nowadays, plant polyphenols enjoy an ever-increasing recognition not only by scientific community but also, and most remarkably, by the general public because of their presence and abundance in fruits, seeds, vegetables and derived foodstuffs and beverages, whose regular consumption has been claimed to be beneficial for human health [3, 5]. Indeed, epidemiological and experimental studies have shown the potential of polyphenols or polyphenolic nutritional sources in reducing the risk of chronic diseases such as cardiovascular diseases [6-10] and cancers [10-14], as well as the risk of degenerative diseases [10, 15, 16]. Altogether these observations led to the current nutritional recommendations to eat five servings of fruits and vegetables per days in order to keep healthy.

A wealth of data, including epidemiological and animal studies, has described the chemopreventive and anticancer properties of polyphenolic compounds, such as resveratrol, curcumin or tea catechins, or polyphenol-rich nutritional sources [13, 14, 17-19]. Nonetheless, recent investigations have highlighted additional mechanisms responsible for direct anti-proliferative and chemotherapeutic properties of polyphenols. Indeed, these compounds can interfere with the initiation, as well as the progression of cancer through the modulation of different cellular events, such as cell cycle arrest by decreasing cyclins or apoptosis induction through

cytochrome c release, activation of caspases and down- or up-regulation of Bcl-2 family members, and inhibition of survival/proliferation signals (AKT, MAPK, NF-κB, etc.). Furthermore, they play an important role in inflammation (COX-2, TNF secretion, etc.), as well as in suppression of key proteins involved in angiogenesis and metastasis [13]. Importantly, it has been established that tumor suppressors like p53 and its analogs are key molecular targets of polyphenols responsible for their pro-apoptotic effect in human and animal cancer models. Here we provide an overview of the molecular mechanisms involved in p53 family proteins modulation by three major and well characterized polyphenolic compounds, resveratrol, curcumin and EGCG.

3. p53 family proteins are chemotherapeutic targets of polyphenols

Since the discovery of p53 in 1979 [20-22] numerous studies have been conducted related to its functions in response to stress and its regulatory mechanisms. p53 is a sequence-specific nuclear transcription factor that binds to defined consensus sites within DNA as a tetramer and represses transcription of a set of genes involved in cell growth stimulation, while activating a different set of genes involved in cell cycle control, like p21. It causes growth arrest providing a window for DNA repair or elimination of cells with severely damaged DNA strands. In some conditions, p53 activation triggers the transcription of pro-apoptotic genes such as Bax or PUMA, as well as the repression of anti-apoptotic genes like survivin [23]. Moreover, p53 can induce transcription-independent apoptosis. This mechanism involves early p53 translocation to mitochondria where it binds to Bcl-2 family proteins, such as Bax, Bak and Bcl-XL, activating cytochrome c release and caspases cascade [24]. Undoubtedly p53 exerts major anti-neoplastic effects and is considered actually as the "guardian of the genome" [25]. Tumor suppressive capabilities of p53 are related to a coordinated regulatory circuit that monitors and responds to a variety of stress signals, including DNA damage, abnormal oncogenic events, telomere erosion and hypoxia [26]. Importantly, in unstressed cells, p53 is latent and is maintained at low level by targeted ubiquitin-mediated degradation related to its interaction with ubiquitine ligases, mainly MDM2 [27]. Regarding the "guardian" functions of p53, mutations of p53 gene or disruptions of p53 coordination such as post-translational inactivation, can disturb the normal physiological balance, and lead to cancer if genome disarrangement reachs a critical value [28]. Indeed, low level of functionnal p53 is a common characteristic of cancer from several localizations including lung, colon, rectum, breast, brain, bladder, stomac, prostate, ovary, liver or lymphoid organs [29]. Somatic p53 missense inactivating mutations are found in approximately 50% of human cancers [30] and this inactivating mutations render the mutant p53 protein unable to carry out its normal function, that is, transcriptional transactivation of downstream target genes that regulate cell cycle and apoptosis [31-33]. On the other hand, p53 pathway can be also inactivated in wild-type (WT) p53-carrying tumors via indirect mechanisms such as MDM2 amplification leading to p53 destabilization [34, 35].

Recently, cDNAs with strong homologies to p53 have been identified and their products were termed p63 and p73 [36-38]. Both proteins are structurally similar and functionally related to

p53, and consequently the entire p53 family may be regarded as a unique signalling network controlling cell proliferation, differentiation and death. Interestingly, in contrary to p53, the role of the other two p53-related proteins in tumor suppression is less obvious, since they are rarely deleted or mutated in cancer, and the respective knockout mice die tumor-free from developmental defects [39-41]. However, increasing number of evidences suggest that both p63 and p73 have a role in tumor suppression. Indeed, different studies indicated that TAp73 and TAp63, the transcriptionally active isoforms, can induce cell cycle arrest, senescence, DNA repair, and apoptosis in response to chemotherapeutic drugs, independently of p53 [42-45]. In addition, even if not mutated, p63 and p73 can be aberrantly expressed in cancer. More particularly, the dominant negative and transcriptionally inactive isoforms ΔNp63 and ΔNp73 are frequently overexpressed in a wide range of tumors, in which they are associated with poor prognosis [46]. Actually, the imbalance in the TAp73/ΔNp73 may be more critical for tumorigenesis and response to chemotherapy than mutations [47]. In summary, despite their differences, the three members of the p53 family may be considered as therapeutic targets for cancer management.

Many *in vitro* studies as well as few *in vivo* studies have shown that resveratrol, curcumin and EGCG, as well as nutritional sources of polyphenols induce overexpression of wild-type p53 (Table 1-4). The p53-related anticancer properties of these three isolated molecules have been extensively evaluated but other polyphenolic compounds such as genistein, luteolin, quercetin, and wogonin have been shown also to upregulate wild-type p53 protein in several cancer cell lines [48-51]. The polyphenol-induced stabilization and expression of wild-type p53 is often associated with a G1 or G2/M phase cell cycle arrest together with transcriptional regulation of target genes such as p21, Bax, PUMA and apoptosis induction [52-56]. The key role of p53 in polyphenol-induced anticancer properties is supported by studies indicating that p53 downregulation counteracts apoptosis triggered by natural products. Indeed, p53 silencing by siRNA abrogate the cytotoxic effect of curcumin in chondrosarcoma cells [57] and genetic invalidation of p53 by shRNA leads to inhibition of EGCG plus luteonin-induced apoptosis of lung cancer cells [58]. In addition, EGCG fails to induce significant cytotoxic effect in p53-null PC-3 prostate cancer cells, but forced expression of p53 in such cell line leads to sensitization to the polyphenolic compound [53]. Indeed, in the later study EGCG induces p53 phosphorylation on Serine 15 and upregulation of p53 and p21 expression together with cell cycle arrest and apoptosis. However the key role of p53 in the anticancer properties of polyphenols is still controversial, especially for curcumin, since many studies have shown its anti-proliferative properties in several p53-mutated or p53-null cancer cell lines (Table 2). For instance, curcumin has significant anti-proliferative effects in two p53-mutated human glioblastoma cell lines, indicating alternative and p53-independent pathway involved in such anticancer properties [59]. Similarly, curcumin reduces glioblastoma cells viability irrespective of p53 mutational status [60]. In this study, curcumin-induced cancer cell death was associated with caspase-3 activity in p53-wild-type cells, but not in p53-mutated cells, indicating that polyphenols can trigger p53- and caspases-independent cell death. p53-independent anticancer properties of polyphenols have been also described in many other cancer cells [61-67]. Interestingly, curcumin reduces the expression of the mutated form of p53 in MDA-MB-231 breast cancer cells together with cell cycle arrest [68], suggesting that a polyphenol-dependent
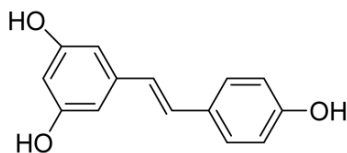
regulatory process can also modulate the expression of a non-functional tumor suppressor. However, despite potential apoptosis induction by polyphenols in absence of functional p53 protein, its wild-type expression makes cancer cells more sensitive to pro-apoptotic effects of polyphenols. Recently, Ferraz da Costa et al. have demonstrated that transient transfection of wild-type p53 in human non-small lung carcinoma cell line H1299 (p53 negative) dramatically increased susceptibility to resveratrol-induced apoptosis [69]. Altogether these data indicate that p53 participates to the cytotoxic effect of polyphenols but also that alternative pathways might be involve in their anticancer properties.

One of this alternative pathway might involve Egr-1, an immediate early-response gene induced by stress, injury, mitogens, and differentiation [70]. Egr-1 regulates the expression of genes involved in the control of growth and apoptosis by transactivating many proteins including p21. One study has shown that transcription of the p21 gene is activated by Egr-1 independently of p53 but under the control of MAPKs in response to curcumin treatment in U-87MG human glioblastoma cells [71]. In addition, the apoptotic effect of resveratrol in colorectal cancer cells as well as EGCG-mediated cytotoxicity in pulmonary cancer cells are also associated with Egr-1 upregulation [72, 73].

Alternatively to p53, its functionally related proteins p63 and p73 might represent targets for polyphenols. Nevertheless only few data are available concerning a potential regulatory effect of polyphenolic compounds on p63 and p73 (Table1-4). Different flavones (luteolin, apigenin, chrysin) and flavonols (quercetin, kaempferol, myricetin) are able to induce cytotoxicity in p53-mutated oesophageal squamous carcinoma cells together with upregulation of p63 and p73 [74]. Similarly, EGCG induces selective apoptosis in multiple myeloma cells with overex-pression of p63 and p73 without any change in the p53 expression level [75], as well as overexpression of p73 in p53-mutated T-lymphocyte leukemic cells [76]. As previously mentioned, different isoforms of p73 have been described and quercetin has been shown to control the subcellular localization of the dominant negative isoform ΔNp73 in melanoma cells expressing wild-type p53. In this model, quercetin caused redistribution of ΔNp73 into the cytoplasm and nucleus, which has been associated with increased p53 transcriptional activity and apoptosis [47, 77]. Beside isolated compounds, more complex sources of polyphenol such as red wine polyphenolic extract or berries-derived product can also modulate p53 and/or p73 expression level, *in vitro* and *in vivo* (Table 4) [78-81]. Interestingly, a synthetic analogue of curcumin increases p73 expression level in two distinct p53-wild-type pancreatic cancer cell lines, BxPC-3 and Colo-357 together with upregulation of pro-apoptotic effector Bax and simultaneous downregulation of the anti-apoptotic protein Bcl-2 [82]. Curcumin itself has been shown to stimulate p53 and also p73 expression in p53-mutated C33A cervical cancer cells [83]. Moreover, EGCG upregulates transcriptional target of p53, in a p53-independent but p73-dependent manner in mouse embryonic fibroblasts [84]. These data suggest that independ-ently of the p53 status (wild-type, mutated or deleted), p73 seems to be involde in the anticancer effect of polyphenolic compounds. Many others studies have shown the potential of polyphe-nols to induce apoptosis of cancer cells in a p53-dependent but also a p53-independent manner (Table 1-4). In summary data concerning the role of tumor suppressors in the polyphenol-induced anticancer effects are inconsistent, probably dependent on the cell type, and conse-

quently remain controversial. Moreover, the molecular mecanisms responsible for p53-family tumor suppressors regulation by polyphenols are only partially elucidated. However some evidences indicate that polyphenols might modulate p53 or p73 expression as well as their stabilization which are under the control of phosphorylation and acetylation levels.

Resveratrol

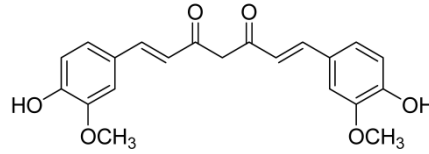| Cancer model	Described effects on TSG (p53/p73)	References
Prostate cancer cells (LNCaP, DU145, p53-mutated CWR22Rv1, p53-null PC-3)	- No change in p53 mRNA, increased expression of p53-p(ser15) and/or p53-ac(lys382) and total p53 protein - p53 translocation to mitochondria - cell cycle alteration and apoptosis induction maintained in p53-mutated cancer cells - potentiation of radiation–induced p53 expression in p53-mutated cancer cells	[135, 158, 164, 165, 175, 176]
Ovarian carcinoma cells (OVCAR-3)	- nuclear accumulation of p53-p(Ser15)	[110]
Breast cancer cells (MCF-7, p53-mutated MDA-MB-231, p53-mutated MDA-MB-435)	- increased expression of p53-p(ser15), p53-p(ser20), p53-p(ser392) and p53-ac(lys382/lys373)	[177-180]
	- no change in total p53 protein expression, p53-independent apoptosis	[178, 181]
	- increased expression of p53 mRNA and total protein	[182-184]
	- no change in p53 mRNA	[185]
	- p53-independent cytochrome c release	[181]
	- increased p53-dependent transcriptional activity	[50]
Colon cancer cells (HCT116, p53-null HCT116)	- increased p53-p(ser15) expression - resveratrol-induced senescence is p53 dependent	[103]
Pancreatic cancer cells (capan-2, colo357)	- upregulation and nuclear accumulation of p53 in both cell line (restoration of wild-type expression)	[186]

Resveratrol
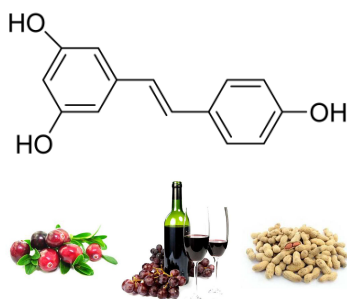
Cancer model	Described effects on TSG (p53/p73)	References
Glioblastoma cells (A172, p53-mutated T98G)	- no change in p53 mRNA	[187]
Hepatocellular carcinoma cells (HepG2)	- no change in p53 mRNA	[185]
Osteosarcoma cells (U-2 OS)	- increased p53-p(ser15) and p53-p(Ser37) expression	[188]
Lung adenocarcinoma cells (A549)	- increased p53-p(ser15) and p53-p(Ser37) expression	[188]
Head and neck squamous cancer cells (UMSCC-22B)	- increased p53-p(ser15) and total p53 expression	[109]
Cervical cancer cells (HeLa)	- increased p53-ac(lys373) and total p53 expression	[185]
Hodgkin lymphoma cells (L-428)	- increased p53-p(ser15) expression	[129]
Follicular lymphoma cells (LY8)	- increased p53-p(ser15) and total p53 expression	[190]
Acute lymphoblastic leukemia cells (MOLT-4)	- increased p53-p(ser15) expression	[189]
Neuroblastoma cells (B65, NUB-7)	- increased p53-p(ser15) and total p53 expression - nuclear translocation of p53	[94, 132]
DMBA-TPA-induced mouse skin tumor ; DEN-induced rat hepatocellular carcinoma	- increased p53-p(ser15) and total p53 expression	[18, 191, 192]
	- increased wild-type p53 and decreased mutated-p53 expression	[193]

Cancer cell lines express wild-type p53 except where otherwise stated; ac(lys.)=acetylated lysine, p(ser.)=phosphorylated serine

**Table 1.** p53 family-related anticancer properties of resveratrol

Curcumin

Cancer model	Described effects on TSG (p53/p73)	References
Breast cancer cells (MCF-7, p53-mutated MDA-MB-231, p53-mutated SkBr3)	- increased expression of p53-p(ser15), no change or increased expression of total p53 -decreased expression of mutated p53	[68, 178, 194-197]
Cervical cancer cells (p53-mutated C33A, Caski)	-increased expression of p53 and p73	[83, 198]
Ovarian cancer cells (HEY, OVCA429, p53-mutated OCC1, p53-null SKOV3, CaOV3, Ho-8910)	-p53-independent cell death	[106]
	-increased expression of p53-p(ser15) -increased expression of p53	[107, 199, 200]
Prostate cancer cells (LNCaP, p53-null PC3)	-increased expression of p53-p(ser15),p53-ac(lys) and total p53 protein -p53-independent cell death -p53 translocation to mitochondria	[95, 100, 201]
Bladder cancer cells (p53 mutated-T-24 and AY-27)	-no change or increased expression of p53	[202, 203]
Erhlich Ascite carcinoma cells	-increased expression of p53-ac(lys373) and total p53	[120]
Colorectal cancer cells (LoVo, HCT116, p53-null HCT116, p53-mutated HT29, p53-mutated Colo205)	-increased expression of total p53	[52, 204]
	-increased expression of p53-p(ser15), p53-p(ser33) and total p53	[64]
	-no change in p53 expression	[173]
	-increased expression of p53-p(ser15) -decreased or unchanged expression of mutated p53	[156, 205]
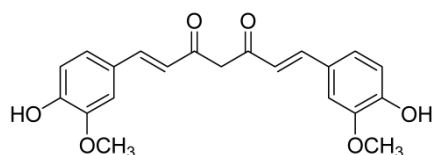
Curcumin

Cancer model	Described effects on TSG (p53/p73)	References
	-cell cycle arrest, senescence and autophagy independent of p53 expression -cytochrome c release independent of p53 expression -increased expression of total p53	[206, 207]
Colitis-associated colorectal cancer in mice	-no change in p53 expression	[17]
Acute lymphoblastic leukemia cells (B6p210, T315I)	-increased expression of total p53	[208]
Chondrosarcoma cells and xenograft in nude mice (JJ012)	-increased expression of total p53 in vitro and in vivo -p53-dependent apoptosis	[57]
Melanoma cells (MMRU, p53-mutated PMWK, B16BL6)	-no change in p53 expression	[209, 210]
Glioblastoma cells (C6, U-87MG, p53-mutated U138MG and U251, DBTRG, T98G, T67)	-p53-independent cell death -unchanged or increased expression of p53	[60, 71, 163, 211, 212]
Neuroblastoma cells (SK-N-AS, NUB-7, p53-mutated SK-N-BE(2))	-p53-independent cell death -nuclear translocation of p53	[65, 94]

Cancer cell lines express wild-type p53 except where otherwise stated; ac(lys.)=acetylated lysine, p(ser.)=phosphorylated serine

Table 2. p53 family-related anticancer properties of curcumin

Epigallocatechin-3-gallate (EGCG)

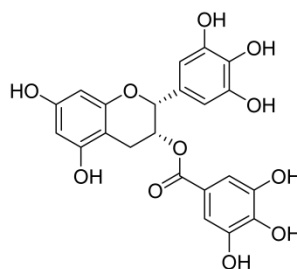| Cancer model	Described effects on TSG (p53/p73)	References
Breast Cancer cells (MCF7, p53-mutated MDA-MB-468)	-increased expression of p53-p(ser15) and total p53 -p53-independent cell death	[213, 214]
Prostate cancer cells (LNCaP, p53-null PC-3, p53-expressing PC-3, PC3-ML, p53-mutated DU-145)	-increased expression of p53-p(ser6), p53-p(ser15), p53-p(ser20), p53-p(ser37), p53-p(ser392) , p53-ac(lys373), p53-ac(lys382) and total p53 -p53-dependent and independent cell death -increased expression of p73	[53, 66, 89, 139, 140, 170, 215]
PC3-ML cells (prostate cancer) xenograft in mice	-increased expression of p53 and p73 (synergistic effect with paclitaxel and docetaxel)	[170]
Cervical cancer cells (Hela)	-increased expression of p53	[216]
Ovarian cancer cells (PA-1, p53-null SKOV3, p53-mutated OVCAR-3)	-p53-independent cell death	[217]
Hepatocellular carcinoma cells (HepG2, p53-null Hep3B)	-increased expression of p53 -p53-independent cytotoxicity	[218, 219]
Colorectal cancer cells (HCT116, p53-mutated HT-29)	-increased expression of p53	[55, 155, 157]
Head and neck squamous carcinoma cells (KB, Hep2, Tu686,	-increased expression of p53-p(ser15) and p53-p(ser37), -decreased expression of p53-p(ser6), p53-p(ser392) -unchanged or increased expression of p53	[101, 172, 220]

Epigallocatechin-3-gallate (EGCG)

Cancer model	Described effects on TSG (p53/p73)	References
686NL, Tu212, Tu177, p53-null M4e)	-p53-dependent cytotoxicity	
Head and neck squamous cell carcinoma syngenic mouse model (SCC VII/SF cells xenograft)	-increased *in vivo* expression of p53-p(ser15)	[101]
Lung cancer cells (A549)	-increased expression of p53-p(ser15) and total p53 -absence of p73 expression -p53-dependent activation of caspases 3/7	[221]
Fibrosarcoma cells (HT-1080)	-increased expression of p53	[222]
Sarcoma xenograft (S180)	-increased *in vivo* expression of p53	[90]
Multiple myeloma cells (INA6)	-increased expression of p63 and 73, unchanged expression of p73	[75]
T lymphocyte leukemic cells (p53-mutated Jurkat, HuT-102, C91-PL, p53-mutated CEM)	-increased expression of p73 -increased expression of p53	[76, 223]

Cancer cell lines express wild-type p53 except where otherwise stated; ac(lys.)=acetylated lysine, p(ser.)=phosphorylated serine

Table 3. p53 family-related anticancer properties of EGCG

Polyphenolic source	Cancer model	p53- and p73-related effects	References
Grape-derived products (red wine, grape seed extract)	C26 colorectal cancer cells xenograft in mice	-increased expression of p53 and p73	[78]
	Human colorectal cancer cells (LoVo, p53-mutated HT29, P53-mutated SW480)	-p53-independent apoptosis	[224]
	Oral squamous carcinoma cells (SCC-25, p53-mutated OEC-MI)	-p53-independent cytotoxicity	[225]
	Leukemia cells (p53-mutated Jurkat)	-increased expression of p73	[80]
	Teratocarcinoma cells (P19)	-increased expression of p53	[174]
	Prostate cancer cells (LNCaP, p53-mutated DU145)	-increased expression of p53-p(ser15) and total p53	[226]
Black and green tea	DMBA-induced mammary tumor in rat	-increased expression of wild-type p53 and decreased expression of mutated-p53	[227]
	3,4-benzopyrene-induced lung carcinoma in rat	-increased expression of p53	[228]
	Ehrlich's ascites carcinoma cell xenograft in mice	-increased expression of p53	[229]
	Oral cells from smoker and non-smoker subjects	-increased expression of p53	[230]
	Patients with high-risk oral premalignant lesions	-no association between p53 expression and clinical response	[231]
	Prostate cancer cells (LNCaP, p53-null PC3)	-increased expression of p53-ac(lys373), p53-ac(lys382) and total p53 -p53-independent apoptosis	[139, 140]
	Colorectal cancer cells (LoVo, p53-mutated HT29)	-p53-independent cytotoxicity	[232]
Berry-derived products (aronia juice, strawberry extract)	Leukemia cells (p53-mutated Jurkat)	-increased expression of p73	[79]
	Breast cancer cells (p53-mutated T47D)	-increased expression of p73	[81]

Cancer cell lines express wild-type p53 except where otherwise stated; ac(lys.)=acetylated lysine, p(ser.)=phosphorylated serine

Table 4. p53 family-related anticancer properties of polyphenolic sources

4. Polyphenols as regulator of p53 expression and localization

Under physiological conditions, the transcriptional activity of p53 is downregulated by three different ways: i) ubiquitin-mediated proteasomal degradation mainly through the action of mouse double minute protein (MDM2), ii) nuclear export leading to a decrease in nuclear level, or iii) transcriptional repression of chromatin. MDM2 is an ubiquitin E3 ligase considered as an oncoprotein because of its activity in promoting p53 ubiquitination and proteasomal degradation. Moreover MDM2 binds to the NH2 terminus of p53 and blocks its transactivational activities [27]. Interestingly, MDM2 promotes also cell cycle progression independently of p53 for instance by modulating the activity of p21 [85]. Then MDM2 itself represents a potential target for new drug with chemotherapeutic properties including polyphenolic compounds [86]. Indeed, curcumin has been identified as an inhibitor of MDM2 expression (Figure 1) *in vitro* and *in vivo* in p53-null and p53-wild-type human prostate cancer cells and this inhibitory effect seems to be related to the inhibition of the PI3K/mTOR pathway [87]. In addition, the curcumin analog EF24, which displays higher potency, increases phosphorylation of p53 together with downregulation of MDM2, which likely leads to p53 overexpression and cytotoxicity in hepatocellular carcinoma cells [88]. Similarly, EGCG reduces MDM2 expression in prostate cancer cells [89], but not in sarcoma cells [90]. Data concerning the effect of resveratrol on MDM2 expression are more controversial since upregulation or downregulation have been observed in different cancer models [91, 92].

As mentioned previously, p53 activity depends upon its expression level but also its subcellular localization. Indeed, p53 displays direct pro-apoptotic effects related to mitochondrial translocation and this pathway works in synergy with transcriptional activation function of p53 dependent upon its nuclear translocation [24, 93]. Therefore, the control of p53 subcellular localization might interfere with p53-mediated cell death. For instance, treatment of neuroblasma cells by either curcumin or resveratrol transiently upregulated p53 expression and induced nuclear translocation of p53, followed by induction of p21 and Bax expression associated with apoptosis [94]. In addition curcumin increases p53 and Bax expression in mitochondrial fraction under the control of the PI3K/Akt pathway in prostate cancer cells followed by caspase-dependent apoptosis [95]. Altogether, these data indicate that polyphenols are able to control not only p53 expression, but also its localization and therefore its proapoptotic activity in cancer cells. However, other post-translational regulatory effects of polyphenols have been also described and related to phosphorylation and acetylation of the tumor suppressor.

5. Polyphenols as regulator of p53 phosphorylation

Phosphorylation of serine/threonine residues are essential for stabilization and activation of p53, the most extensively studied being serine 15 (Ser 15). These phosphorylation sites are mainly concentrated in the N-terminal transactivation domain and in the C-terminal regulatory domain [96]. Recent data about p53 phosphorylation induced by resveratrol,
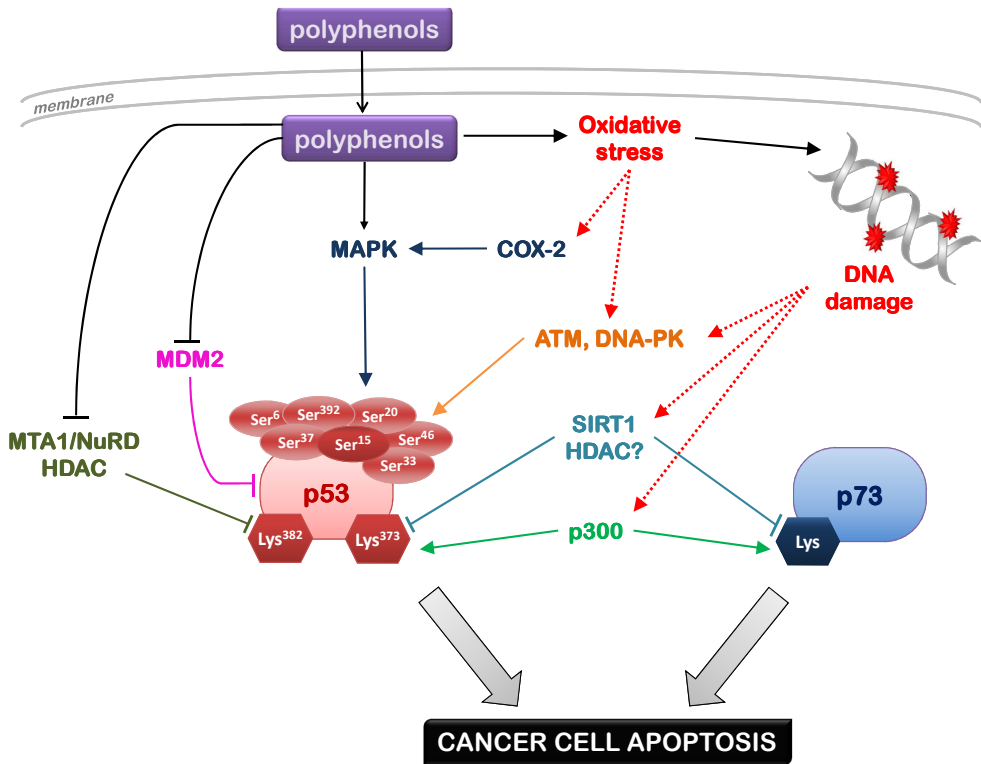
Figure 1. Overview of p53- or p73-mediated pro-apoptotic effects of polyphenols in cancer cells. Polyphenols likely induce intracellular oxidative stress and DNA damage with subsequent activation of kinases (MAPK, ATM, DNA-PK) responsible for p53 phosphorylation. Simultaneously, and also in response to DNA damage, acetylation of p53 or p73 have been described due to enhanced acetylase activity from p300 and/or to reduced deacetylase activity from SIRT1 or HDAC. In addition, p53 expression has been shown to be under the control of MDM2 as well as MTA1/NuRD, both factors being downregulated by polyphenols. Phosphorylation and acetylation, together with MDM2 inhibition result in p53, and to a less extend, p73 stabilization and sustained expression which activate cell death cascade in cancer cells.

curcumin or EGCG, *in vitro* and *in vivo*, are summarized in Tables 1, 2 and 3 as well as in Figure 1. The DNA damage is one of the main signals relayed to p53 subsequently activated by phosphorylation at serine residues that are the target of ataxia-telangiectasia mutated (ATM), ataxia telangiectasia and Rad3 related (ATR) and DNA-dependent protein kinase (DNA-PK) [97]. The DNA damage response could be activated by chemotherapeutic drugs, UV or oxidative stress [98, 99], but activation of this pathway by polyphenols remains controversial. Watson et al. investigated the pro-apoptotic effect of curcumin which is similar in p53+/+ (wild-type) and p53-/- (knockout) HCT116 colorectal cancer cells. Moreover, they demonstrated the ability of this polyphenol to induce up regulation of p53-p(Ser 15) and total p53 without any change in the expression level of ATM, ATR or DNA-PK. In contrast, curcumin enhances p38, JNK and ERK1/2 phosphorylation in both p53+/+ and p53-/- HCT116 cell lines; this suggests that the cytotoxic effects of curcumin are

independent of the DNA-damage/ATM/ATR/DNA-PK pathway but associated with Mitogen-Activated Protein Kinases (MAPKs) activities [64]. On the other hand, treatment of LNCaP prostate cancer cells or HCT116 colorectal cancer cells with curcumin induces the phosphorylation of ATM, histone H2AX (a marker of DNA damage) and p53 at Ser 15 together with increased expression of p53, suggesting p53 activation through the DNA damage/ATM pathway [52, 100]. The importance of ATM in polyphenols-induced cytotoxicity is also supported by recent data showing that EGCG lose the ability to trigger p53 phosphorylation at Ser 15 in absence of ATM [101]. In addition, genistein induced p53 phosphorylation at Ser 6, 9, 15, 20, 46 and 392 in the ATM-proficient human lymphoblastic cell lines, but not in ATM-deficient cell lines, indicating a key role of ATM kinase activity for polyphenol-induced p53 activation [102]. Moreover, stimulation of the ATM/p53 pathway by polyphenols like resveratrol has been shown to also participate in senescence of cancer cells [103]. On the other hand, quercetin strongly induced DNA-PK expression, p53 phosphorylation and apoptosis in melanoma cells, suggesting that other kinases might be activated by polyphenols [77].

Alternatively, MAPKs such as ERK1/2, p38 or JNK have been involved in p53 activation and phosphorylation [104, 105]. Therefore, the potential MAPKs/p53-dependent activation of apoptosis by polyphenols has been investigated. As previously mentioned, curcumin induces phospho-p38, phospho-ERK1/2 and phospho-JNK in colorectal as well as ovarian cancer cells [64, 106, 107]. In addition, it has been shown that resveratrol- or luteolin-induced apoptosis depends on the activities of ERK1/2, JNK and p38 kinase which target p53 phosphorylation at Ser 15 [49, 108]. An alternative pathway which implicates cyclooxygenase (COX)-2 activity and expression has been decribed by Lin et al. They have shown that resveratrol-induced apoptosis of human head and neck squamous cancer cells or human ovarian carcinoma cells is associated with p53 phosphorylation at Ser 15 and that both processes are downregulated by pERK1/2 and COX-2 specific inhibitors [109, 110]. Recent investigations indicate that ERK and p53 regulate each other and that ATM controls their interaction [104]. Therefore polyphenols might likely trigger p53 activation through ATM and MAPKs complementary pathways.

6. Polyphenols as regulators of p53 and p73 acetylation

Functions of p53 and p73 are also regulated by acetylation on different lysine (Lys) residues. These posttranslational covalent modifications occur in response to DNA damage in the close vicinity of the oligomerization domain. The main Histone Acetyl Transferases (HATs) responsible for these modifications include p300, CREB-Binding Protein (CBP), P300/CBP-Associated Factor (PCAF) and Tat-Interactive Protein of 60 kDa (TIP60) [38]. As a consequence of its acetylation, p53 is stabilized by excluding ubiquitination on the same site and acetylation also promotes p53 transcriptional activity [96]. In comparison to p53, only few data are available concerning p73 interaction with HAT and acetylation. It has been established that p300 can acetylate p73 in response to DNA damage, but p300 can also behave as a co-activator of p73 independently of its HAT activity [111]. Importantly, the level of p53 or p73 acetylation

seems to be a major way of regulation for the tumor suppressors function since deacetylated p53 and p73 are compromised in their ability to induce cell cycle arrest and apoptosis [91, 112].

The transcriptional coactivator p300 is a large multidomain protein that possesses histone acetyl-transferase ability [113]. Together with its homolog CBP, p300 mediate transcription through binding to transcriptional activators such as JUN, E1A, NF-κB, as well as to the p53 family members and they have been involved in human diseases including cancers [114]. Recent studies indicate that the transcriptional activity of p53 and p73 in response to genotoxic stress is regulated by its interaction with p300 [115-117]. Indeed, it has been established that interaction between p73 and p300 acetyl-transferase promotes first p73 stability and then its transcriptional activity.

Narayanan et al., have suggested that resveratrol-induced apoptosis of prostate cancer cells is mediated by transcriptional activation of p300 which subsequently acetylates and stabilizes p53 [118]. Similarly in breast cancer cells, resveratrol enhanced p300 expression and interaction with the phosphorylated form of p53 by a MAPK-dependent mechanism [119]. Interestingly, p53-p300 interaction fails to occur in doxorubicin-resistant cells, but curcumin pre-treatment could restore this interaction. Consistently, curcumin also restored drug-induced p53 acetylation (lysine 373) and p53-dependent transcription of Bax, PUMA, and Noxa in resistant cells leading to their apoptosis [120]. Therefore, polyphenols-induced acetylation of p53 by p300 might represent a key molecular mechanism for the cytotoxic properties of these natural compounds in cancer cells including chemoresistant cells (Figure 1).

Acetylation level of tumor suppressors is dependent upon the balance between acetylation and deacetylation reactions. Indeed, deacetylation of p53 or p73 by SIRT1 (silent information regulator 1), a member of the sirtuin Histone DeACetylase (HDAC) class III family, prevents p53-mediated transactivation of cell cycle inhibitor p21 and pro-apoptotic factor Bax, allowing promotion of cell survival after DNA damage and ultimately tumorigenesis [121]. Members of the Silent Information Regulator family (SIRT or sirtuins) are evolutionary conserved NAD-dependent protein deacetylases and adenosine diphosphate (ADP)-ribosylases. There are seven identified isoforms (SIRT1-7) that differ in their subcellular localization (cytoplasmic, mitochondrial or nuclear), substrate specificities and functions [122]. The founding member of this class of deacetylases, SIRT1 (homolog of yeast silent information regulator, Sir2), is the most widely studied sirtuins. SIRT1 has been associated with aging processes as well as a variety of human diseases such as metabolic syndrome, inflammation, neurodegeneration and more recently cancer [123, 124]. SIRT1 can deacetylate a variety of histones as well as a number of non-histone substrates, the first identified of these non-histone substrates being p53 (Lys 382-p53). The SIRT1 activity on p53 results in repression of p53-dependent apoptosis in response to DNA damage and oxidative stress [125, 126]. SIRT1 deacetylates also other tumor suppressors such as p73 [91]. Then SIRT1 has been considered as an oncogenic protein because of its role in inactivating tumor suppressors such as p53, p73 but also PTEN [127], and/or activating other oncogenic proteins like N-Myc [128]. Nevertheless, the oncogenic potential of SIRT1 has been controversial and, depending on the context, SIRT1 might also act as a tumor suppressor [122]. However, inhibition of the oncogenic potential of SIRT1 is likely able to

induce apoptosis by counteracting the deacetylation of p53 or p73 and other key factors such as FOXO3a [91, 125, 129].

In 2003, resveratrol was the top hit in a screen designed to identify activators of sirtuin enzymes [130] and was subsequently shown to extend lifespan in yeast. However following experiments led to confusing data suggesting that resveratrol might not be a direct activator of SIRT1 [131]. Regardless of the controversy about its mode of action, resveratrol has been confirmed to have numerous health benefits, including anticancer properties. Nevertheless, the role of SIRT1 in the anti-proliferative and pro-apoptotic effects of resveratrol on cancer cells is still unclear. Indeed, in neuroblastoma cells, resveratrol-induced apoptosis was associated with a reduced expression of SIRT1 as well as up-regulation of the acetylated and active form of p53, but the pre-treatment of cancer cells with SIRT1 enzymatic inhibitors such as sirtinol or nicotinamide has no cytotoxic effect suggesting that resveratrol-induced apoptosis is independent of SIRT1 activity [132]. In the opposite, siRNA-mediated downregulation of SIRT1 in lymphoma cells decreased the resveratrol-induced apoptosis, indicating in this case a critical role of SIRT1 in polyphenol-mediated cancer cell death [133]. Interestingly, Frazzi et al., have recently described anti-proliferative effect of resveratrol associated with downregulation of SIRT1 expression and activity together with upregulation of acetylated-Lys 373-p53, the active form of p53, and total p53 overexpression [129]. All together, these data suggest that, in the context of cancer cells, resveratrol might be an inhibitor, instead of an activator, of SIRT1 functions (Figure 1). However, only few data are available concerning potential regulation of SIRT1/p53 pathway by other polyphenolic compounds [134]. Therefore additional investigations are needed to further understand the role of SIRT1 in polyphenols-mediated anticancer effects.

On the other hand, resveratrol has been shown to enhance p53 acetylation and apoptosis in prostate cancer cells through alternative pathways. Indeed, resveratrol caused down-regulation of MTA1 protein, leading to destabilization of MTA1/NuRD complex thus allowing acetylation/activation of p53 [135]. Metastasis-associated protein 1 (MTA1) is part of the nucleosome remodelling deacetylation (NuRD) complex involved in global and gene-specific histone deacetylation, alteration of chromatin structure and transcriptional repression [136, 137]. This complex, which also contains Histone DeACetylase (HDAC)1 and HDAC2, plays an essential role in governing deacetylation of histones but also non histone proteins, such as p53 [138]. In addition, green tea polyphenols have been shown to behave as HDAC class I inhibitors which results in p21 and Bax expression irrespective of p53 status in prostate cancer cells [139, 140]. Moreover HDAC inhibition by EGCG is associated with p53 acetylation in p53-wild-type LNCAP prostate cancer cells suggesting an increase of p53 halftime and binding to p21 and Bax promoters as previously described [141]. The mechanism by which HDAC inhibition could induce apoptosis in absence of functional p53 in p53-null PC3 prostate cancer cells might be related to interaction with p73 pathway as previously suggested [142, 143] or direct regulation of p21 promoter activation [144]. Similar HDAC inhibition by curcumin has been also seen in prostate cancer cells [145]. However the exact role of HDAC inhibition in polyphenol-induced apoptosis of cancer cells remains to be elucidated especially *in vivo*.

7. Role of oxidative stress and DNA damage in p53/p73 regulation by polyphenols

Polyphenolic compounds have been extensively described as anti-oxidant molecules with the capability to scavenge reactive oxygen species (ROS), which include radical oxygen and nitrogen species such as O2- (superoxide anion), HO· (hydroxyl radical), NO· (nitric oxide radical), ONOO- (peroxinitrite anion) and H_2O_2 (hydrogen peroxide), as well as oxidatively generated free radicals RO· and ROO· from biomolecules like lipids, proteins or nucleic acids (DNA and RNA) [3, 146]. Polyphenols are not only able to quench the ROS but also to regulate directly the oxidative stress-mediated enzyme activity, therefore reducing the formation of ROS. These anti-oxidant properties have been linked to the polyphenol-mediated reduction of chronic disease risk including cancer chemoprevention [13, 147]. Indeed, redox changes are often reported as important inducer of neoplastic transformation as well as chemoresistance. Cerutti et al. identified for the first time in 1985 the close relationship between pro-oxidant conditions and cancer development [148]. More than twenty years later, accumulated evidences indicate that the non-physiological alterations of the intracellular redox state could be considered as a hallmark of tumor biology. Indeed, redox changes have been involved in several key events of carcinogenesis such as self-sufficiency in growth signals [149, 150], resistance to apoptosis [151, 152], sustained angiogenesis [153, 154], autophagy and invasiveness. However, recent findings also suggest that this redox changes might be exploited as therapeutic strategy to selectively kill tumor cells.

Recently and unexpectedly, it has been established that various and structurally different (flavonoids or non-flavonoids) polyphenols are able to induce ROS (mainly superoxide anions or hydrogen peroxide) formation in cancer cells and for some of them to activate the DNA-damage response pathway [51, 79, 80, 95, 102, 103, 155-158]. Heiss et al. have also shown that the resveratrol-induced senescence in colon cancer cells is dependent upon an increased formation of ROS and the subsequent phosphorylation of p53 on the Ser15, suggesting a relationship between polyphenol-induced oxidative stress and p53 activation [103]. On the other hand, curcumin and wogonin induce ROS production and cause cytotoxicity in p53+/+ and p53-/- cancer cells [56, 64], indicating that ROS formation is an event independent of p53 and might be an earlier step in the cell death pathway. This hypothesis is supported by the study showing ROS in cancer cells as earlier as 20 minutes after the beginning of wogonin treatment. Moreover, in the same study, the subsequent up-regulation of p53 (maximal activation at 16 hours) is significantly inhibited by anti-oxidants such as N-Acetyl-Cysteine. Importantly, most of the studies did not investigate the possible alternative role of p73 in p53-mutated cells, but we and others have shown that in p53-mutated or p53-deficient cells, a polyphenolic compound (EGCG) or source (red wine polyphenolic extract, polyphenol-rich aronia juice) strongly induced oxidative stress-mediated up-regulation of p73 and apoptosis [79, 80, 84]. Further reports also supported that p53-family tumor suppressors regulation might be related to oxidative stress [159].

Interestingly, the cytotoxic effects induced by curcumin or its analogue HO-3867 were reduced in non-cancerous cells as well as the ROS formation in comparison to human ovarian cancer

cells. This suggests that the specific pro-oxidant activity of polyphenols in cancer cells might explain the selective anticancer properties of these compounds, sparing healthy normal cells [107, 160]. Similarly, EGCG increased preferentially ROS formation, p53 and p21 expression and cytotoxicity in colorectal cancer cells but not in human embryonic kidney cells and normal human lung cell line [157].

Oxidative stress is one of the major conditions that damages DNA, acting as a mediator of environmental stressors such as UV- and X-rays irradiation, drugs, and of metabolic imbalance [161]. Since p53 might be regulated by the redox environment [162], especially by the ROS/DNA damage pathway, it has been proposed that polyphenol-mediated anticancer effects are related to a ROS/DNA damage/p53 pathway (Figure 1). Indeed polyphenol-induced DNA damage and apoptosis have been demonstrated with various compounds such as curcumin in glioblastoma and prostate cancer cells [100, 163], resveratrol in prostate cancer cells [164, 165], EGCG in lung cancer cells and xenograft in mice [166], wogonin in glioblastoma and prostate cancer cells [51, 56], and luteolin in lung and head and neck cancer cells [58]. Therefore the current molecular mechanism of the anticancer properties of polyphenols might involve selective ROS formation together with DNA damage in cancer cells. Thus, this process might lead to the regulation of several pathways (ATM/DNA-PK, MAPKs, p300, SIRT1, HDAC, see Figure 1), and ultimately to the expression and stabilization of p53-family tumor suppressors triggering programmed cell death.

8. Therapeutic perspectives

Recent investigations have demonstrated additional or synergistic effects when polyphenols are combined with chemo- or radiotherapy. Indeed, resveratrol induces synergistic apoptosis with 5-fluorouracile [167]. Similar observations have been made with curcumin associated with doxorubicin, cisplatin, gemcitabine or radiation for cell death induction of glioblastoma cells and prostate cancer cells [60, 87]. More importantly, curcumin and its analogue, HO-3867 sensitized doxorubicin-resistant ascite carcinoma cells and breast cancer cells as well as cisplatin-resistant ovarian carcinoma cells together with enhanced p53 expression [107, 120, 168, 169]. Similarly, EGCG displayed synergistic upregulation of p53 and p73 as well as anticancer properties with taxanes (paclitaxel and docetaxel) *in vitro* but also *in vivo* in prostate cancer models [170, 171]. Because all of the previously mentioned drugs demonstrated the ability to induce DNA damage, it is likely that polyphenols might amplify these damages leading therefore to synergistic effects. Surprisingly, EGCG has also synergistic effects with targeted therapy such as erlotinib (inhibitor of epidermal growth factor receptor) to induce p53 phosphorylation on Ser15 and expression together with apoptosis [172].

Interestingly, curcumin also ameliorated oxaliplatin-induced chemoresistance in colorectal cancer cells without significant effect on p53 expression [173]. Similarly, curcumin and EGCG sensitized glioma cells *in vitro* and *in vivo* to chemotherapeutic drugs and also to radiation in a p53-independent manner [163]. These data suggest that polyphenols can effectively circumvent resistance of cancer cells to chemotherapy, but likely through a p53-independent pathway.

Numerous *in vitro* studies have demonstrated the cytotoxic effects of polyphenols by using micromolar concentrations which are much higher than current chemotherapeutic drugs under development. However, polyphenols still keep their potential as chemotherapeutic drug, firstly because of their activity on chemo- or radioresistant cancer cells and secondly because of their very low toxicity on healthy tissues giving them a large therapeutic index. Indeed, many recent *in vitro* studies have highlighted the selective pro-apoptotic properties of polyphenols or analogues with no or low cytotoxic effect on non-cancer healthy cells, such as endothelial cells, cardiomyocytes, lymphocytes, chondrocytes, ovarian cells, prostate and mammary epithelial cells, astrocytes, or neurons [54, 57, 60, 79, 95, 107, 158, 163, 169, 174]. Interestingly, the selective pro-apoptotic effect of curcumin in breast cancer cells is associated with an increased expression of p53, whereas p53 is only slightly upregulated in normal mammary epithelial cells, suggesting a selective activation of p53 pathway in cancer cells sparing normal cells [54]. Moreover, *in vivo* treatment with polyphenolic compounds or products in tumor model such as cancer cells xenografts induced a significant inhibition of tumor growth together with a very good tolerance for healthy tissues, including heart, liver, kidney, lung and haematopoietic tissue [60, 78]. However, more animal studies and human clinical trials are now necessary to clearly determine whether polyphenols or their natural nutritional sources are safe and efficient to treat cancer.

9. Concluding remarks

The present literature review has summarized the results of recent studies focusing mainly on the p53-related anticancer properties of three major polyphenolic compounds (resveratrol, curcumin and EGCG). Despite highly active research in this area, the data are still controversial concerning the possible key role of the tumor suppressor p53 in the polyphenol-mediated apoptosis of tumor cells. However, according to the emerging evidences suggesting that polyphenols might alternatively regulate also the structurally- and functionally-related tumor suppressors such as p73, these natural compounds might be considered as general executors of the p53 family-mediated programmed cell death in cancer cells. Importantly, the selective anticancer properties of polyphenols are maintained even when p53 is mutated or absent, as well as when cells are resistant to current therapies. However, further investigations are still mandatory to better understand the underlying molecular mechanism *in vitro* as well as *in vivo* before a potential clinical development.

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START-GAP/DLC Family Proteins: Molecular Mechanisms for Anti-Tumor Activities

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Additional information is available at the end of the chapter

1. Introduction

The Rho family of GTPases belongs to the superfamily named "Ras-like" proteins, which consists of over 150 varieties in mammals [1]. Significant progress has recently been made in understanding the biological functions mediated by this family of small (~21 kDa) G proteins (guanine nucleotide-binding proteins). Rho GTPases affect crucial biological processes such as transcriptional regulation, cell cycle progression, apoptosis and membrane trafficking [2, 3]. Thus far, a total of 23 Rho proteins have been identified [4], among which RhoA, Rac1 and Cdc42 are characterized in detail. Rho GTPases are also involved in the cytoskeleton formation of the cell via the regulation of actin dynamics [5, 6]. RhoA induces stress fiber formation and focal adhesion assembly, thereby regulating cell shape, attachment and motility, whereas Rac1 promotes extension of lamellipodia and membrane ruffling [7]. Cdc42 has been shown to play a role in the formation of filopodia [8].

Like other small G proteins of the Ras-like protein family, Rho GTPases act by switching between an inactive GDP-bound and an active GTP-bound form, with the latter form capable of interacting with a myriad of downstream effectors (so far, more than 70 proteins have been identified [4]) to be activated by them. The activation of Rho GTPases is stimulated by guanine nucleotide exchange factors (GEFs) that exchange GDP for GTP and is inhibited by GTPase-activating proteins (GAPs) that hydrolyze the GTP to GDP [9]. Rho GTPases are also negatively regulated by guanine nucleotide dissociation inhibitors (GDIs), which bind to the GDP-bound form and not only prevent nucleotide exchange, but also remove Rho GTPases from the plasma membrane to the cytoplasm [4, 10, 11]. Taken together, cell morphology requires spatiotemporally restricted regulation of Rho GTPases through these regulatory proteins. Nonetheless, detailed insight into regulation of Rho GTPases has not been provided.

The effect on the wide spectrum of biological functions suggests the involvement of Rho GTPases and their regulators in cancer progression. Findings from extensive in vitro and in vivo studies shows that deregulated signaling of Rho GTPases may lead to tumorigenesis [12] and thus Rho GTPases are taken as potential targeting candidates for cancer therapy [13]. Since no constitutively active Rho mutants have been reported in human cancers, it is likely that aberrant Rho GTPase signaling in malignancy is caused by the alterations of their regulators [4, 12]. Extensive studies so far have revealed that regulators of Rho proteins are over- or underexpressed in various types of human cancer cells [14-17]. The most common alteration reported for Rho regulators in cancer is inactivation of RhoGAPs, especially of the START-GAP/DLC family RhoGAPs [18]. "START" stands for "steroidogenic acute regulatory protein (STAR)-related lipid transfer" and "DLC" stands for "Deleted in Liver Cancer (DLC)," a gene (or its product) found to be commonly deleted in liver tumors. The START-GAP/DLC family proteins have become the focus of attention on their roles in tumorigenesis. This type of genetic loss was also found in a number of other cancers [17, 19-21].

START-GAP1/DLC1 was originally cloned from rat cDNA library as a binding partner of phospholipase C-δ1 (PLCδ1) [22]. It has been shown that the C-terminal region of START-GAP1/DLC1 and the PH domain of PLCδ1 are responsible for the interaction [23, 24]. START-GAP1/DLC1 enhances the activity of PLCδ1, which generates two second-messengers, inositol 1,4,5-trisphospate (Ins(1,4,5)P_3) and diacylglycerol via hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2). Ins(1,4,5)P_3 is accepted by receptors on ER, resulting the elevation of intracellular calcium concentration, whereas diacylglycerol acts as the activator for PKC [25, 26]. Indeed, microinjection of START-GAP1/DLC1 into the cytosol elevated intracellular calcium concentration [23].

As mentioned in detail in the following sections, each member of the START-GAP/DLC multidomain protein family contains one GAP domain for Rho GTPases. Overexpression of START-GAP1/DLC1 in cultured cells was first to demonstrate induction of drastic morphological changes accompanied by the disruption of actin stress fibers and elevation of intracellular Ca^{2+} concentrations [23]. START-GAP1/DLC1 was therefore originally designated ARP (adaptor for both Rho and PLC) [22], but later the name p122RhoGAP (or just p122) was used to avoid confusion with another ARP (actin related proteins) [23, 24, 27, 28]. We have then introduced the new name, START-GAP1, based on the characteristic domain structure of this Rho GTPase family [29-32]. Meanwhile the antioncogenic properties of the protein family have been revealed and the DLC nomenclature was introduced [21]. In this chapter, the combination of both the structure- and function-based nomenclatures is used throughout, since the use of either of them would not be appropriate to reflect the fundamental properties of the protein family accurately.

In the following sections we will focus on the structure, localization and expression-function relationship of the START-GAP/DLC family proteins in physiological conditions and in human diseases.

2. The START-GAP/DLC gene family

In mammalian genome, there are three genes encoding structurally-related RhoGAPs containing the START domain (Figure 1). There are three groups of START domain-containing RhoGAP proteins in vertebrates, while a worm (*Caenorhabditis elegans)* or a fly (*Drosophila melanogaster)* possesses only one START-containing RhoGAP.
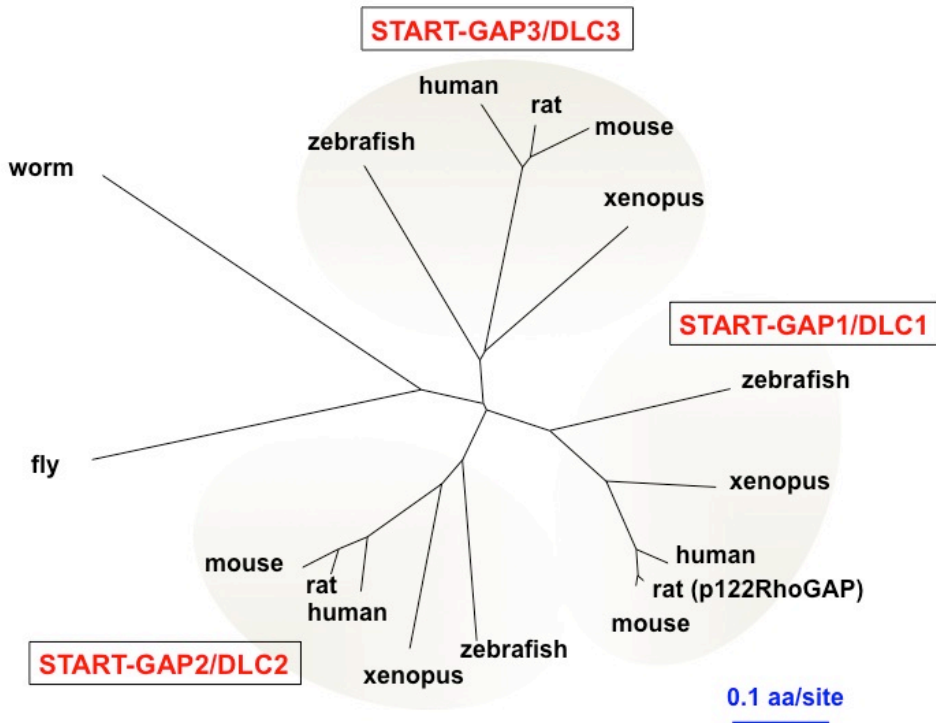
Figure 1. A phylogenetic analysis of the START domain-containing RhoGAPs generated with a "Treeview" after CLUSTAL W analysis (http://clustalw.ddbj.nig.ac.jp/). Modified from Kawai et al. [31].

2.1. Human START-GAP/DLC genes and their expression in various tumor cells

There are about 70 human genes encoding RhoGAPs that share a conserved GAP domain and are capable of switching off the Rho signal [33]. The *START-GAP1/DLC1* (also named as STARD12 or ARHGAP7) gene is localized on chromosome 8p21-22 and encodes a 1,091-amino acid protein with a molecular mass of 122 kDa. Using the quantitative RT-PCR method, Ko et al. have reported that START-GAP1/DLC1 is widely expressed in normal tissues, with high abundance in the lung and ovary, and moderately in the thyroid, spleen, intestine and kidney [34]. START-GAP1/DLC1 has also been known as a tumor suppressor gene product. It is

frequently underexpressed or not expressed in several tumor cells and inhibits cell growth, invasion and metastasis [35, 36]. Studies have indicated that downexpression of START-GAP1/DLC1 either by genomic deletion or DNA methylation [37] is associated with a variety of cancer types including lung [38], breast, prostate, kidney, colon, uterus, ovary, and stomach [38] [39] [40]. These phenotypes require the GAP activity of START-GAP1/DLC1 [35].

Negative regulation of the Rho/Rho-kinase (ROCK)/myosin light chain (MLC) pathway in hepatocellular carcinoma (HCC) cell lines by START-GAP1/DLC1 was shown to be RhoGAP-dependent [23, 41, 42]. The RhoGAP defective mutant failed to inhibit stress fiber formation in HCC lines [41], whereas the overexpression of START-GAP1/DLC1 resulted in morphological change with disruption of actin stress fibers [23, 42]. Using various cancer cell models, START-GAP1/DLC1 was shown to inhibit cell proliferation, suppress cell migration and invasion, and induce apoptosis [35, 36, 41, 43-45]. Restoration of START-GAP1/DLC1 expression in metastatic cell lines has been shown to cause the inhibition of cell migration and invasion as well as a significant reduction in metastases in nude mice [45].

Underexpression of START-GAP1/DLC1 was associated with either heterozygous deletions of the *START-GAP1/DLC1* gene or hypermethylation of the gene promoter region [17, 37, 46-49]. This protein is therefore thought to be under the epigenetic regulation for expression.

The *START-GAP1/DLC1* gene is transcribed from two different promoters, resulting in transcripts encoding three isoforms [47]. To date, most of studies on START-GAP1/DLC1 have focused on the so-called isoform 2. Low et al. have recently identified a new isoform of START-GAP1/DLC1, isoform 4 (DLC1-i4), using 5'-RACE method [50]. This novel isoform encodes a 1,125-amino acid protein with distinct N-terminus as compared with other three isoforms. Similar to them, DLC1-i4 is expressed ubiquitously in normal tissues and immortalized normal epithelial cells, suggesting a role as a major START-GAP1/DLC1 transcript. Differential expression of the four START-GAP1/DLC1 isoforms, however, is found in tumor cell lines: Isoform 1 (the longest isoform) and isoform 3 (short and probably nonfunctional) share a promoter and are silenced in almost all cancer and immortalized cell lines, whereas isoform 2 and isoform 4 utilize different promoters and are frequently downregulated. Isoform 4 is significantly downregulated in multiple carcinoma cell lines, including nasopharyngeal, esophageal, gastric, breast, colorectal, cervical and lung carcinomas. Ectopic expression of DLC1-i4 suppresses tumor cell colony formation. Differential expression of the isoforms suggests interplay in modulating the complex activities of START-GAP1/DLC1 during carcinogenesis.

There are two additional members of the START-GAP/DLC family. *START-GAP2/DLC2* (or *STARD13*) gene is located on chromosome 13q12 [51] and *START-GAP3/DLC3* (or *STARD8/KIAA0189*) gene is located on the X chromosome at q13 band [31, 52]. The *START-GAP2/DLC2* encodes a 1,113-amino acid protein with a molecular mass of 125 kDa, whereas the protein product of the START-GAP3/DLC3 transcript has 1,103 amino acids with a molecular mass of 121 kDa. START-GAP2/DLC2 has a broad tissue distribution, with the highest levels in the brain, heart and liver [15, 53]. Human START-GAP2/DLC2 protein shares 51% identity and 64% similarity to human START-GAP1/DLC1 at the level of the amino acid sequence [51]. Introduction of human START-GAP2/DLC2 into mouse fibroblasts suppress Ras signaling and

cell transformation in a GAP dependent manner, suggesting the role of START-GAP2/DLC2 for growth suppression and carcinogenesis [51].

START-GAP3/DLC3 is also detected in a variety of human tissues with high abundance in the lung, kidney and placenta [53].

Following the findings of dysregulation of the START-GAP1/DLC1 gene function in a variety of solid tumors, downregulation of START-GAP2/DLC2 and START-GAP3/DLC3 genes was also shown to be involved in human cancer development.

START-GAP2/DLC2 was found to be downregulated in breast, lung, ovarian, renal, uterine, gastric, colon, rectal, and liver tumors [15, 52, 54]. The comparison of *START-GAP1/DLC1* and *START-GAP2/DLC2* gene expression in the same cell lines revealed that START-GAP2/DLC2 is more frequently downregulated than START-GAP1/DLC1 in HCC cell lines [15]. Moreover, the overexpression of START-GAP2/DLC2 suppresses cell proliferation, motility and anchorage-independent growth in the human hepatoma cell line, HepG2 [55]. START-GAP2/DLC2 was also reported to have an inhibitory effect on the growth of breast cancer cells in vitro [56].

Decreased START-GAP3/DLC3 expression in primary tumors from kidney, lung, uterine, ovary and breast has been reported [52]. Kawai et al. have demonstrated that START-GAP3/DLC3 serves as a GAP for both RhoA and Cdc42 in in vitro assays. Furthermore, the overexpression of START-GAP3/DLC3 in HeLa cells disrupts actin stress fibers and changes cell morphology in a GAP-dependent manner [31]. Ectopic expression of START-GAP3/DLC3 in human breast and prostate cancer cell lines inhibits cell proliferation, colony formation and growth in soft agar [52].

The structures of the three human START-GAP/DLC transcripts are depicted in Figure 2.
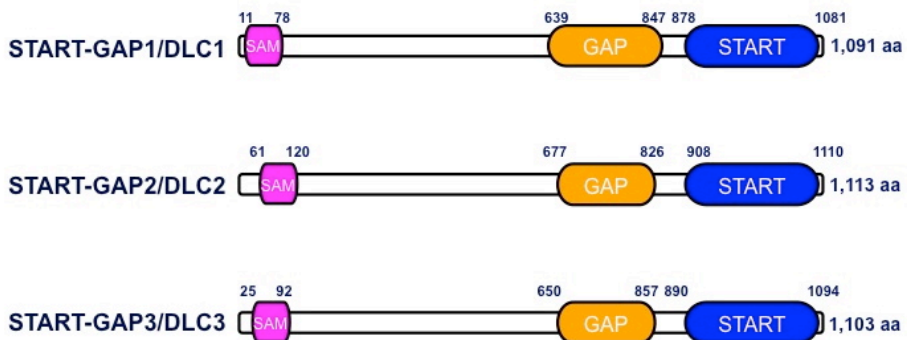
Figure 2. Schematic representation of the START-GAP/DLC family proteins. Each member of the family comprises of three distinct domains, namely the sterile α motif (SAM), RhoGAP (GAP) domain and START (steroidogenic acute regulatory protein (STAR)-related lipid transfer) domain.

2.2. Gene knockout studies of START-GAP/DLC proteins

Using a mouse model a gene knockout study of START-GAP1/DLC1 has been carried out [57]. The mouse *START-GAP1/DLC1* gene was inactivated by homologous recombination. Mice heterozygous for the targeted allele were phenotypically normal, but homozygous mutant embryos did not survive beyond 10.5 days postcoitum. Cultured fibroblasts from START-GAP1/DLC1-deficient embryos displayed alterations in the organization of actin filaments and focal adhesions [57]. In addition, a gene knockdown of START-GAP1/DLC1 in the background of c-*myc* overexpression promotes the formation of liver tumors [58].

Although the START-GAP1/DLC1 gene deficient mice were embryonic lethal, deletion of the *START-GAP2/DLC2* gene from mice resulted in survival to adulthood, indicating that the gene, unlike the *START-GAP1/DLC1* gene, was dispensable for embryonic development [59]. Neither did the authors observe a higher incidence of liver tumor formation in the *START-GAP2/DLC2* gene knockout mice. Nevertheless, they reported smaller phenotype with less formation of adipose tissue [59].

To the best of our knowledge, no reports describing the gene knockout study of the START-GAP3/DLC3 gene are currently available.

2.3. Homologs of the START-GAP/DLC family

As mentioned earlier, there are homologs of the mammalian START-GAP/DLC family proteins in invertebrates in the BLAST database. A *Drosophila* ortholog of START-GAP1/DLC1, *Crossveinless-c* (*Cv-c or RhoGAP88C*), was identified in search for genes that regulate *Drosophila* morphogenesis [60]. The function of *Cv-c* has been revealed to be a key regulator for unidirectional growth of dendritic branches of the fly via downregulating the activity of Rho1, the *Drosophila* Rho GTPase [61]. In the *Cv-c* mutant, two subclass of multidendritic sensory neurons formed dorsally directed branches; however, dendritic branches had a difficulty in growing along the anterior–posterior (A–P) body axis, suggesting that *Cv-c* contributes to sprouting and subsequent growth of the A–P-oriented branches through negative regulation of Rho1 [61]. Thus *Cv-c* plays a key role in directional dendritic growth presumably via its RhoGAP activity, localizing the GAP activity to sites undergoing cytoskeleton rearrangements during morphogenesis.

3. Structure and function of the domains

The START-GAP/DLC family proteins are composed of multi-domain structures. START-GAP/DLC proteins have three distinct domains: The sterile α motif (SAM) localized at its N-terminus, a conserved RhoGAP (GAP) domain in the middle and the steroidogenic acute regulatory (StAR)-related lipid transfer (START) domain at the C-terminus [51-53]. (Figure 2)

Although the START-GAP/DLC family proteins contain a potential lipid-binding START domain, the proteins are produced in soluble forms. The intracellular localization of these proteins, therefore, is determined by their specific interactions with target proteins resided at

various cellular structures. Each domain of the START-GAP/DLC family proteins may contribute to different subcellular localization patterns.

3.1. The GAP domain

The biological activity of START-GAP1/DLC1 is mainly executed by the GAP domain (~150-200 amino acid), which promotes the hydrolysis of GTP bound to the Rho GTPases. This catalytic activity is mediated by the 'arginine-finger' present in the GAP domain [62]. START-GAP1/DLC1 and START-GAP3/DLC3 contain a conserved 'arginine-finger' at position 677 and 688, respectively [52, 53]. In in vitro assays both the full-length START-GAP1/DLC1 and the isolated GAP domain reveal activity on RhoA, RhoB and RhoC, to a lesser extent on Cdc42, and no activity on Rac1 [17, 32, 63]. By inactivating these small GTPases, START-GAP1/DLC1 affects cell morphology and control actin cytoskeletal remodeling [22, 23]. Similar to START-GAP1/DLC1, both START-GAP2/DLC2 and START-GAP3/DLC3 contain a RhoGAP domain and exhibit the GAP activity for RhoA and Cdc42 but not Rac1 in vitro [31, 32, 51].

3.2. The sterile α motif (SAM)

The SAM domain (~70 amino acids) has been found in signaling proteins (e.g., p53 related proteins p73 and p63, Eph-related tyrosine kinases and Ets transcription factors) [64, 65]. The SAM domain is thought to act as a protein interaction module via homo- or hetero-oligomerization with other SAM domains [64]. As an example, the EphA2 receptor that plays key roles in many physiological and pathological events including cancer has the SAM domain. Recently, a structural study of the EphA2 receptor SAM domain has validated structural elements relevant for the heterotypic SAM-SAM interactions: two SAM domains interact with a head-to-tail topology characteristic of several SAM-SAM complexes [66]. The SAM domain even interacts with RNA [67].

Structural studies of the SAM domain of START-GAP2/DLC2 have suggested that it binds to lipids such as phosphatidylglycerol [68]. Nevertheless, we know little about exact roles of the SAM domain in START-GAP/DLC function. Kim et al. have shown that the expression of the amino-terminal domain of START-GAP1/DLC1 acts as a dominant negative and profoundly inhibits cell migration by displacing endogenous START-GAP1/DLC1 from focal adhesions [69]. The SAM domain of START-GAP1/DLC1 may serve as an autoinhibitory domain for intrinsic RhoGAP catalytic activity. Eukaryotic elongation factor-1A1 (EF1A1) was found to be a target of the SAM domain of START-GAP1/DLC1 [70]. EF1A1 is involved in protein synthesis [71] and also in transporting β-actin mRNA [72]. EF1A1 is a regulator of cell growth and the cytoskeletal network controlling the actin network through its G-actin-binding activity [73], F-actin-bundling activity [74] and by stabilizing microtubules [75]. EF1A1 is overexpressed in various human cancers, including pancreas, lung, prostate, breast and colon cancers [71]. The SAM domain of START-GAP1/DLC1 adopts a four-helix fold similarly to the SAM domain of START-GAP2/DLC2, but it utilizes a unique motif on a hydrophobic surface to bind directly to EF1A1 [70]. Importantly, the SAM domain is necessary for START-GAP1/DLC1 to translocate EF1A1 to the membrane periphery and ruffles upon fibroblast growth factor stimulation, acting as an auxiliary oncogenic switch to the GAP domain [70].

3.3. The START domain

The START domain (~210 amino acids) is a well-conserved lipid binding domain, which is primarily found in proteins that transfer lipids between organelles and are involved in lipid metabolism as well as in modulation of signaling events involved in lipid processing [76, 77]. The mammalian START domain protein family is well characterized and is composed of 15 members that are classified into 6 subfamilies based on the sequence and ligand specificity: STARD1/3 and STARD4/5/6 subfamilies bind cholesterol and oxysterols, STARD2 (PCTP: phosphatidylcholine transfer protein)/7/10/11(CERT: ceramide transfer protein) subfamily binds phospholipids and ceramides/sphingolipids, STARD14 binds possibly fatty acids. They all may have roles in non-vesicular lipid transport. STARD14/15 subfamily consists of proteins with the thioesterase activity such as the Acyl-CoA thioesterase (ACOT) family proteins. The START-GAP/DLC family proteins fall into the STARD8/12/13 subfamily [76, 77]. Lipid binding properties of START-GAP1/DLC1 (STARD12), START-GAP2/DLC2 (STARD13) and START-GAP3/DLC3 (STARD8) have not well characterized yet.

Among these proteins, STARD1 (or steroidogenic acute regulatory protein, StAR) and STARD3 (or metastatic lymph node 64 kDa protein, MLN64) appear to be the most well characterized [78]. Both STARD1 and STARD3 bind cholesterol [79], and are also known to play a role in lipid transport into mitochondria [80, 81]. In particular, STARD1 localizes to the mitochondria and stimulates the translocation of cholesterol from the outer to the inner mitochondrial membranes [79]. It has been suggested that STARD1 is an essential component in steroid hormone production in steroidogenic cell [82, 83]. It is noteworthy, therefore, that START-GAP2/DLC2 has been found to localize in mitochondria and was found in proximity to the lipid droplets through the START domain [84]. Future research is required in order to establish the lipid ligand of the START domain of START-GAP2/DLC2 and clarify whether the START domain plays a role in mitochondrial lipid transport. START-GAP2/DLC2 also mediates ceramide activation of phosphatidylglycerolphosphate (PGP) synthase and drug response in Chinese hamster ovary cells [85].

The crystal structures for the START domains of human STARD3/MLN64 (PDB entry: IEM2) [86] and murine STARD4 (PDB entry: 1JSS) [87] were the first to be solved and showed an α-β helix-grip fold with a nine-stranded anti-parallel β-sheet forming a U-shaped hydrophobic cleft that binds the ligand and is flanked by N- and C-terminal α helices. The C-terminal α helix is proposed to serve as a 'cap' to the ligand-binding site, with lipid access to the binding pocket requiring a conformational change in the START domain and movement of the C-terminal helix. To date, the crystal structures for a limited number of the START domain-containing proteins were solved: human STARD1/StAR (PDB entry: 3P0L) [88], human STARD5 (PDB entry: 2R55) [88], human STARD2/PCTP (PDB entry: 1LN1) [87], STARD11/CERT (PDB entry: 2E3R) [89], human STARD13/START-GAP2/ DLC2 (PDB entry: 2PSO) [88] and human STARD14 (PDB entry: 3FO5) [88]. The data confirm the basic helix-grip fold structure across the five mammalian subfamilies that defines this family of proteins [88].

3.4. The FAT region

Between the SAM and GAP domains there is a long unstructured region (~190 amino acids) termed the FAT (focal adhesion targeting) region, due to the fact that its presence determines of focal adhesion localization of the START-GAP/DLC proteins [27]. START-GAP1/ DLC1, START-GAP2/DLC2 and START-GAP3/DLC3 are recruited to focal adhesion sites via their FAT sequence, which binds to the *Src* homology 2 (SH2) domains of focal adhesion proteins and interacts with tensins [32, 43, 90]. The same region of START-GAP1/DLC1, however, has been found to possess the ability to interact with the PTB domain of tensin2 [30, 91, 92] and tensin1 [43]. The role of the interaction with the PTB domain in the localization of START-GAP1/DLC1 to focal adhesions remains controversial and requires further investigation. Using pull-down assay, Kawai et al. reported the interaction between the other members of the START-GAP/DLC family, START-GAP2/DLC2 and START-GAP3/DLC3, and tensin2 PTB [30].

Since the START-GAP/DLC family proteins are rich in serine residues, especially in their FAT regions, it is natural to propose that these proteins could be phosphorylated by the AGC (protein kinases A, G, and C) family protein kinases. Indeed, a numerous potential phosphorylation motifs by these kinases can be found in the START-GAP/DLC family proteins. Protein kinase B (PKB or Akt) is among the member of the kinase family. Since PKB/Akt plays an essential role in the actions of growth factors as well as the regulation of many other cellular processes, such as apoptosis and anoikis, neuronal development and degeneration, and the cell cycle [93], its involvement in function of the START-GAP/DLC family proteins is argued [94].

Hers et al. have demonstrated that Ser[322] in the FAT region of rat START-GAP1/DLC1 is phosphorylated upon insulin stimulation of intact cells and that this site is directly phosphorylated in vitro by PKB/Akt and ribosomal S6 kinase (RSK1), another member of the AGC family of protein kinases [95], suggesting the phosphorylation via both the PKB/Akt and MAPK kinase (MEK)/extracellular signal-regulated kinase (ERK)/RSK pathways by growth factors. In other words, this site has the potential to integrate the activities of two different signal transduction pathways in a manner dependent on the cellular context. As Ser[322] falls within the FAT region, its phosphorylation may be involved in regulating the targeting of STASRT-GAP1/DLC1 to focal adhesions. However, despite the profound morphological changes, both S[322]A and S[322]D mutants showed similar localizations to focal adhesions as the wild-type STASRT-GAP1/DLC1. The function of the phosphorylation in signaling events downstream of PKB/Akt, such as GLUT4 translocation, the activation of RhoA effectors and cellular transformation, therefore awaits further studies.

A recent report by Ko et al. [96] has also postulated a central role of PKB/Akt phosphorylation of human START-GAP1/DLC1 in the regulation of its tumor suppressive activity, but it argues against the results obtained from the previously-mentioned study on rodent STASRT-GAP1/ DLC1. Although human START-GAP1/DLC1 has three characteristic phospho-PKB/Akt substrate motifs, the authors showed that only Ser[567] was phosphorylated by PKB/Akt. Only active PKB/Akt was able to interact with STASRT-GAP1/DLC1. Since phosphorylated START-GAP1/DLC1 forms a more stable interaction with PKB/Akt, there seems to be a cooperative

increase in binding. Furthermore, Ko et al. showed that unphosphorylated START-GAP1/DLC1 is sufficient to suppress proliferation and anchorage-independent cell growth. Using a *ras* transformed, *p53*-deficient murine hepatoma line, the authors demonstrated that only wild-type START-GAP1/DLC1 or a phosphorylation-dead mutant ($S^{567}A$) was able to inhibit tumor formation in nude mice, whereas the $S^{567}D$ mutant, simulating constitutive phosphorylation, did not inhibit tumor growth. PKB/Akt, suggesting that all START-GAP/DLC family members may share common mechanisms of post-translational regulation, also phosphorylated human START-GAP2/DLC2 in the corresponding motif.

The central region of START-GAP1/DLC1 containing the FAT region was reported to target caveolae by interacting with caveolin-1 [28, 92].

4. Regulation of intracellular localization

Generally, members of the START-GAP family, START-GAP1/DLC1, START-GAP2/DLC2 and START-GAP3/DLC3, START-GAP/DLCs do not localize evenly in the cytoplasm. Rather, they are localized in the specialized place in intracellular spaces. All three members are localized to focal adhesions of attached cells. A conserved region among them (the FAT region), responsible for targeting to focal adhesions, has now been identified. It is now established that the tensin family, which is the major component of the focal adhesion complex, is responsible for recruiting the START-GAP/DLC family proteins to focal adhesions. Nevertheless, many proteins are now revealed to interact with START-GAP/DLCs. Vinculin, another member in the focal adhesion complex, can also interacts with the START-GAP/DLC family proteins. In addition, PLCδ1 interacts with all three isoforms of START-GAP/DLCs and make a molecular complex in lipid rafts upon stimulation with extracellular stimuli. Moreover, 14-3-3 binds to START-GAP1/DLC1. Binding of 14-3-3 proteins often sequesters the target protein in a particular subcellular compartment and the release of 14-3-3 proteins then allows the target to be relocated. Since START-GAP1/DLC1 has a nuclear localization signal (NLS) sequence and found in the nucleus, it is suggested that it also interacts with importins.

Thus expected function of the START-GAP/DLC family proteins varies from one cell type to next, depending on the spatiotemporal regulation according to the binding proteins. Nevertheless, as a whole, they regulate cell shapes and motility via remodeling of actin cytoskeleton.

4.1. Focal adhesion targeting via interaction with tensin and vinculin

START-GAP1/DLC1 is localized in focal adhesions via the FAT region located in its N-terminal half and interacts with tensin family proteins, that constitutes focal adhesion components. Evidences that the interaction between START-GAP1/DLC1 and tensin2 occurs in a PTB domain-dependent manner have been provided. It was revealed that FAT3, the third subregion of the FAT region divided into five (39 amino acids), binds directly to the PTB domain of tensin2 [30]. This interaction does not require protein phosphorylation, since the interaction was detected with proteins expressed in bacterial expression system.
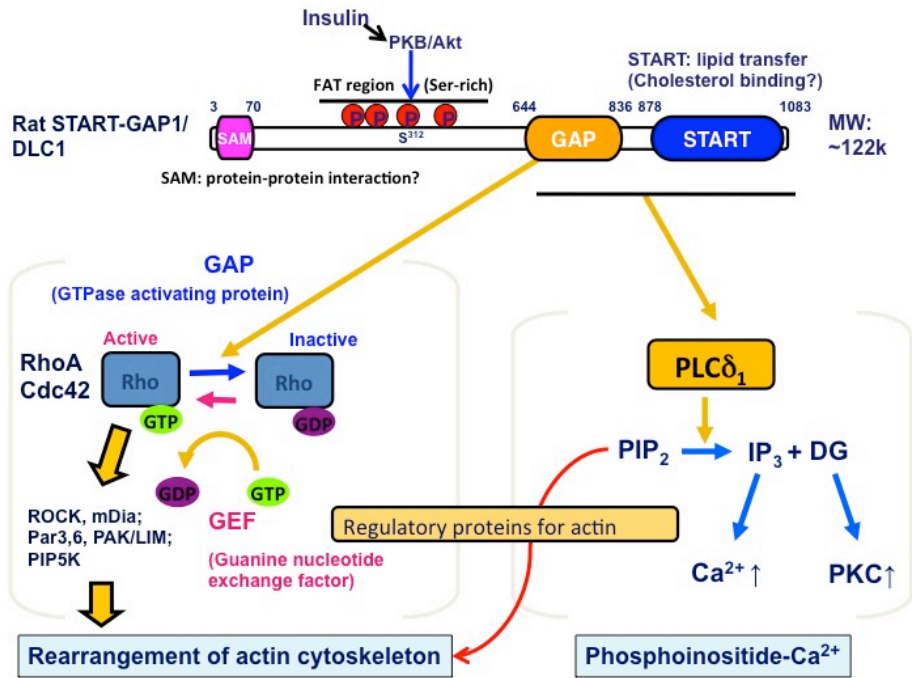
Figure 3. START-GAP1/DLC1 contributes to at least two signaling pathways. START-GAP1/DLC1 consists of about 11 hundred amino acids (for rat: 1,083 and for human: 1, 093) and contain the sterile α motif at the N-terminus, which is known to function in protein-protein interaction. In the C-terminal half there are "GAP domain" followed by the START domain, which is generally thought as a lipid binding or transfer domain. The GAP domain shows a GTPase-activating function, specific for Rho family GTPases, RhoA and Cdc42 but not Rac1 [31], converting "the active, GTP-bound form" to "the inactive, GDP-bound form." The GAP activity of START-GAP1/DLC1 therefore may be implicated in control of the cytoskeleton, cell polarity and cell migration. As for phosphoinositide signaling, one of the downstream effectors of RhoA is PIP5K that generates PtdIns(4,5)P_2. So by inhibition of activated Rho, PtdIns(4,5)P_2 generation is expected to be reduced. The C-terminal half of START-GAP1 is responsible for stimulation of PLCδ1 and breaks down PtdIns(4,5)P_2. This not only causes the generation of two second messengers, Ins(1,4,5)P_3 and diacylglycerol, but also alters the activity of several PtdIns(4,5)P_2-dependent actin-regulating proteins. As a whole, in microenvironment surrounding active START-GAP1/DLC1, RhoA and Cdc42 are inacitvated and a loss of PtdIns(4,5)P_2 is expected. The region between the SAM and GAP domains is rich in serine residues and does not fall on any known protein domains and has been thought to form disordered conformation.

START-GAP2/DLC2 and START-GAP3/DLC3, as well as STRT-GAP1/DLC1, bind to the PTB domain of tensin2, presumably due to the presence of highly conserved residues in the center of FAT3. Deletion of this sub-region abrogates the interaction with the tensin PTB domain. The tensin2 PTB domain seems to determine the subcellular localization of FAT3. Nevertheless, our study with deletion mutants revealed that FAT3 is essential but not sufficient for the focal adhesion localization of START-GAP1/DLC1. These results suggest that the interaction between the tensin PTB domain and FAT3 contributes to START-GAP1/DLC1 localization but only partially. Other factors could affect the START-GAP1/DLC1 localization.

Amino acid sequence of START-GAP3/DLC3 contains a segment similar to the START-GAP1/DLC1 tensin binding site ([353]STYDNL[358]) and full-length START-GAP3/DLC3 was shown to bind the SH2 and PTB domains of tensin1 [43].

We noticed that tensin2 does not always colocalize with START-GAP1/DLC1 and that this interaction was insufficient for targeting START-GAP1/DLC1 to focal adhesions. We thereby explored if there is another molecule that interacts with START-GAP1/DLC1 by the GST pull-down assay using GST-START-GAP1/DLC1 transfected HeLa cells. As a result, we found that START-GAP1/DLC2 can bind with the C-terminus (730-1066) of vinculin. Moreover, START-GAP1/DLC1 and vinculin were found colocalized in foal adhesions. The domain in START-GAP1/DLC1 required for interaction with vinculin was narrowed down to residues 460-470 including an LD motif, a motif in paxillin required for interaction with vinculin. A deletion mutant and point mutants of this motif were fully localized to focal adhesions, although they lost binding ability to vinculin. It is likely that when START-GAP1, vinculin and tensin2 form a stable complex, they are localized to focal adhesions.

PtdIns(4,5)P_2 has an important role in regulating cytoskeleton assembly by inducing conformational changes in actin binding proteins such as vinculin and talin [97]. Thus, START-GAP1/DLC1 could influence cytoskeletal dynamics by altering local PtdIns(4,5)P_2 levels by activating PLCδ1 in the vicinity of focal adhesions as well as by regulating Rho GTPase activity there.

4.2. Raft localization via interaction with PLCδ1

The C-terminal region of START-GAP1/DLC1 covering the GAP and START domain is known to interact with PLCδ1, enhancing its activity [22]. Although START-GAP1/DLC1 binds with PLCδ1, it is not usually localized at the plasma membrane where PLCδ1 targets itself through its PH domain under unstimulated conditions.

Caveolae are plasma membrane domains that appear as flask-shaped invaginations at the cell surface and are enriched in cholesterol and sphingolipids. Yamaga et al. have shown that endogenous START-GAP1/DLC1 was found to sediment with caveolin-1 in low density cholesterol-rich membrane fractions [28], and both endogenous and exogenous START-GAP1/DLC1 were co-immunoprecipitated with caveolin-1 [28, 92]. Since multiple functions have been proposed for caveolae [98] and caveolin-1 has been found in membrane subdomains other than caveolae, including focal adhesions [98] the physiological significance of the interaction between START-GAP1/DLC1 and caveolin-1 requires further investigation.

In their previous study [28], Yamaga et al. provided supportive evidences for START-GAP1/DLC1 localization in caveola, which is cholesterol-enriched membrane microdomain, using an expression system of GFP-tagged proteins, an immunoprecipitation assay and a sucrose density gradient centrifugation analysis of cell lysates. GFP-tagged START-GAP1/DLC1 was observed as patch-like structures at the cell surface and in the cytoplasm of attached cells. The patches were dependent on the levels of the membrane cholesterol and co-localized with caveolin-1. START-GAP1/DLC1 interacts with caveolin-1 in vivo and is fractionated into low-density caveolin-enriched membrane fractions. These results support the idea that START-GAP1/DLC1 is targeted to caveolae via binding to caveolin-1. Yamaga et al. also found the C-

terminal half of START-GAP1/DLC1 was responsible for its patch-like distribution. The authors found amino acid sequences that resemble putative caveolin-binding motifs (ΦΧΦΧΧΧΧΦ, ΦΧΧΧΧΦΧΧΦ or ΦΧΦΧΧΧΧΦΧΧΦ), where Φ is an aromatic residue and X is any amino acid [99] in the N-terminal region ([14]WLRVTGFPQY[23] and [93]WTFQRDSKRWSRLEEFDVF[111]) and in the GAP domain ([690]YVNYEGQSAY[699] and [725]FLQIYQY[731]) of START-GAP1/DLC1. Since GFP-tagged START-GAP1/DLC1–534ΔC does not seem to reside in caveolin-1-containing membranes, the sequences in the N-terminal region may not function as caveolin-binding motifs. Either (or both) the sequence(s) in the GAP domain, however, could be important for caveolin-1 binding and therefore responsible for the distribution of START-GAP1/DLC1. It is therefore possible that the RhoGAP domain of START-GAP1/DLC1 contributes not only to the catalytic activity but also to the intracellular localization of START-GAP1/DLC1. The exact roles of the N-terminal region have to be clarified. GFP-tagged START domain was also observed as patches, but unlike GFP-tagged full-length protein or GFP-tagged GAP domain, it was just partially co-localized with caveolin-1. The patch-like localization of GFP-tagged START domain was similar to the distribution of cholesterol visualized by filipin, a fluorescent molecule that specifically binds to free cholesterol. The result suggests that the START domain can associate with cholesterol but it is not sufficient to recruit START-GAP1/DLC1 to caveolin-1-enriched membrane microdomains.

Recruitment and activation of PLCδ1 in lipid rafts by a muscarinic agonist was examined using PC12 cells [24]. Sucrose density gradient centrifugation analyses of cell lysates revealed that only a small amount of PLCδ1 was recovered in low-density membrane fractions. The amount, however, significantly increased when cells were treated with a muscarinic agonist carbachol. Immunoprecipitation studies demonstrated that carbachol also enhanced the interaction between PLCδ1 and START-GAP1/DLC1, which is constitutively localized in lipid rafts. The PH domain of PLCδ1 is likely responsible for the interaction. Since carbachol elevates intracellular Ca^{2+} levels in PC12 cells, Yamaga et al. next examined whether a rise of intracellular Ca^{2+} levels participates in the carbachol-induced raft recruitment of PLCδ1. After treatment with a Ca^{2+} ionophore ionomycin PLCδ1 was also translocated to lipid rafts in PC12 cells. Chelating extracellular Ca^{2+} by EGTA did not inhibit the carbachol-induced translocation of PLCδ1 to lipid rafts, whereas treatment of cells with thapsigargin to block the intracellular Ca^{2+} mobilization inhibited its translocation. These results suggest that PLCδ1 is recruited to lipid rafts and binds to preexisting START-GAP1/DLC1 in Ca^{2+} dependent manner, and this process can be triggered by external stimuli activating GPCRs (Figure 4).

Although the physiological function and significance of START-GAP1/DLC1 in caveolar localization remain unclear, it appears that caveolae are one of the compartments whereby START-GAP1/DLC1 may exhibit its tumor-suppressive role and possibly vasoregulatory role which will be mentioned in Section 7, and affect the cytoskeletal reorganization by its RhoGAP activity.

4.3. START-GAP2/DLC2 localization in mitochondria

Apart from focal adhesion localization, START-GAP/DLCs could be localized in mitochondria. Ng et al. has disclosed that START-GAP2/DLC2 is targeted to mitochondria through the

START domain using Huh-7 hepatoma cells [84]. They could observe the expression of ectopic START-GAP2/DLC2 in the cytoplasm especially in a punctate structure, suggesting that START-GAP2/DLC2 was concentrated in cytoplasmic speckles. It is noteworthy that the localization patterns of full-length START-GAP2/DLC2, START-GAP2/DLC2-ΔSAM lacking the N-terminal SAM domain and START-GAP2/DLC2-START, that consists of only from the START domain were very similar, indicating that the START domain is responsible for the intracellular localization. Analysis of the amino acid sequence has revealed that no noticeable localization signal in the START domain. START-GAP2/DLC2-START was in dot-like structure throughout the cytosol, although some cells showed aggregates in the perinuclear region, while START-GAP2/DLC2-ΔSTART, a mutant lacking the START domain, was found to distribute homogenously in the cytoplasm. Notably, almost all START-GAP2/DLC2 signals overlapped those of mitochondria, but not all mitochondria were targeted by START-GAP2/DLC2. They also biochemically fractionated mitochondrial fraction from myc-tagged START-GAP2/DLC2 expressed Huh-7 cells and found that it was found in both the cytoplasmic and mitochondrial fraction. Taken together, the START domain plays a pivotal role in intracellular localization and function of START-GAP2/DLC2. Whether these results could also apply for endogenous START-GAP1/DLC1 or START-GAP3/DLC3 in other cell types awaits future experiments.

4.4. START-GAP2/DLC2 localization around lipid droplets

In addition to demonstrate the mitochondrial localization of START-GAP2/DLC2, Ng et al. also examined whether START-GAP2/DLC2 and/or the START domain of START-GAP2/DLC2 can interact with intracellular lipids [84]. Since localization pattern of START-GAP2/DLC2 and its START domain are very similar, they examined whether lipophilic dyes such as Nile red and Sudan III overlap with signal of the START domain of START-GAP2/DLC2. The speckles containing the START domain were localized around the lipid droplets stained by Nile red. Nevertheless, the two signals did not overlap substantially with each other, despite that a major portion of the START domain was found to be in proximity to the lipid droplets. The authors suggested that the START domain of START-GAP2/DLC2 likely serves a lipid related function in the cell and it targets START-GAP2/DLC2 to areas proximal to the lipid droplets [84].

4.5. START-GAP1/DLC1 localization in the nucleus

Recently, using lung carcinoma cells Yuan et al. showed that START-GAP1/DLC1 harbors a functional bipartite nuclear localization signal (NLS) in serine-rich domain in the FAT region, which works together with the GAP domain to mediate START-GAP1/DLC1 nuclear import and subsequent apoptosis [38]. The potential NLS sequence was found as amino acids [415]RRENSSDSPKELKRRNS[431] including a pat7 NLS spanning residues [423]PKELKRR[429] [100]. A deletion mutant START-GAP1/DLC1-Δ372 lacking the N-terminus 372 amino acids accumulates in the nucleus, suggesting the presence of nuclear export signal (NES) in this region, whereas mutants with disruption of this NLS sequence could only localize in the cytosol.

The function of START-GAP1/DLC1 in the nucleus remains to be defined. Some Rho GTPases have C-terminal polybasic region that could be function as an NLS [101]. Therefore these GTPases may serve as substrates for START-GAP1/DLC1 in the nucleus. In the nucleus START-GAP1/DLC1 may also interact with non-GTPase substrates such as PLCδ1 [22], which is under nuclear import-export equilibrium and accumulate in the nucleus under specific phases of the cell cycle [102, 103] or extracellular stimuli that causes aberrant increase in intracellular Ca^{2+} [103-106]. Nuclear translocation of START-GAP1/DLC1 was proposed to be associated with apoptosis by a yet unknown mechanism [38].

START-GAP1/DLC1 interacts with 14-3-3 proteins, resulting inhibition of the GAP activity and block nucleo-cytoplasmic shuttling [100]. In GST pull-down assays, START-GAP1/DLC1 interacted with all 14-3-3 isoforms except 14-3-3σ. The other six 14-3-3 isoforms are ubiquitously expressed, readily form homo- and heterodimers, and could therefore potentially participate in the regulation of START-GAP1/DLC1 function. 14-3-3 proteins often regulate cellular processes by modulating target protein localization. The pat7 NLS spanning residues of START-GAP1/DLC1, [423]PKELKRR[429], was demonstrated to be masked by phorbolester-induced phosphorylation and the 14-3-3 interaction. Inactivation of this NLS by exchange of critical arginine residues impaired but did not prevent nuclear import of START-GAP1/DLC1. This suggests the presence of another NLS that contributes to START-GAP1/DLC1 nuclear shuttling.

Taken together, it was suggested that START-GAP1/DLC1 functions both as a cytoplasmic and nuclear tumor suppressor.

5. Negative regulation of carcinogenesis not dependent on the RhoGAP activity

5.1. RhoGAP activity-dependent pathway

As mentioned earlier, the START-GAP/DLC family proteins may exert their suppressive function by decreasing the levels of active, GTP-bound Rho proteins or inhibiting the GDP-GTP cycling process, affecting cytoskeletal remodeling, cell shape, motility, proliferation and apoptosis. Nevertheless, molecular mechanisms through which START-GAP/DLC family proteins are capable of suppressing cell motility and cell growth are still unclear.

Holeiter et al. have shown that enhanced migration of cells lacking START-GAP1/DLC1 is dependent on the Rho effector, Dia1, and does not require the activity of ROCK [107]. Leung et al. provide evidence for a key mechanism through which the START-GAP/DLC family proteins act as tumor suppressors [108]. In this study, the authors demonstrated that the expression of START-GAP2/DLC2 is involved in the inactivation of the Raf/MEK/ERK/RSK pathway, which is crucial for cell proliferation. This inhibitory activity of START-GAP2/DLC2 is attributed to RhoGAP function [108].

By binding to PLCδ1, START-GAP1/DLC1 enhances the hydrolysis of PtdIns(4,5)P_2, which interacts with a variety of actin regulatory proteins that affect the actin cytoskeleton [22, 23,

28]. In addition to the PLCδ1 activation and the modulation of RhoGAP activities, proper focal adhesion localization and interaction with tensins have been demonstrated to be essential for the growth-suppression function of START-GAP1/DLC1 [43, 90, 92, 109].

START-GAP1/DLC1 overexpression induces a significant reduction of stress fibers and filopodia, as well as membrane blabbing and cellular decomposition, nuclear condensation or fragmentation in NCI-H358 cells known as highly sensitive cells to tumor suppression functions of START-GAP1/DLC1 [110]. These effects were due to the collapse of actin cytoskeleton during apoptosis [111].

To overcome the embryonic lethality of homozygous START-GAP1/DLC1 knockdown [57], Zender et al. combined in vivo RNAi and a 'mosaic' mouse model of HCC to address the impact of the loss of START-GAP1/DLC1 on liver carcinogenesis [112]. Genetically modified liver progenitors (p53$^{-/-}$ cells infected with a retrovirus expressing *c-myc* and another expressing a START-GAP1/DLC1 shRNA) were transplanted into the liver of syngenic mice to assess their ability to generate tumors in situ. In contrast to control shRNA, START-GAP1/DLC1 shRNA accelerates the formation of liver tumors, which mimics aggressive HCC. Conversely, reintroduction of START-GAP1/DLC1 in hepatoma cells co-expressing oncogenic Ras results in a dramatic reduction of tumor growth in situ. This study demonstrates that START-GAP1/DLC1 loss, when combined with other oncogenic lesions, efficiently promotes the development of HCC.

Activation of RhoA, and thereby of its downstream effector ROCK, is both necessary and sufficient to promote HCC in vivo. Therefore, START-GAP1/DLC1, due to its RhoGAP activity, is capable to antagonize the activities of RhoA and its downstream effectors in HCC [58, 113, 114]. Moreover, RhoA is required for tumorigenesis induced by the loss of START-GAP1/DLC1 and constitutively active RhoA mimics loss of START-GAP1/DLC1 in promoting HCC in the 'mosaic' mouse model [58].

START-GAP1/DLC1 was also implicated in tumor metastasis. The expression levels of START-GAP1/DLC1 mRNA are significantly lower in highly invasive tumors than in less invasive ones [115]. Restoration of START-GAP1/DLC1 expression in vitro reduces the migration and the invasiveness of HCC cells [35, 41, 113]. This mechanism also seems to be dependent on RhoGAP activity and its downstream effectors ROCK and MLC [42].

Finally, reexpression of START-GAP1/DLC1 in HCC cells downregulated the expression of osteopontin and matrix metalloproteinase-9, which are found overexpressed in most primary metastatic liver tumors [114]. START-GAP1/DLC1 restoration also suppresses the distant dissemination of cells from subcutaneous tumors developing after inoculation of HCC cell lines in mice. This process is also dependent on the RhoA activity and reorganization of actin cytoskeleton in tumor cells [114].

5.2. RhoGAP activity-independent pathway

Angiogenesis is another form of cancer development. In addition to its direct effect via activation of Rho pathway, START-GAP1/DLC1 is responsible for regulation of angiogenesis in an indirect manner [116]. START-GAP1/DLC1 negatively regulates angiogenesis in a

paracrine fashion. START-GAP1/DLC1 silencing promoted pro-angiogenic responses through vascular endothelial growth factor upregulation, accompanied by the accumulation of hypoxia-inducible factor 1 and its nuclear localization.

6. Stability of START-GAP/DLCs

Stability of proteins may also contribute to the expression levels of the START-GAP/DLC family proteins. Generally, degradation of proteins is controlled by ubiquitination-dependent and independent proteolysis. START-GAP1/DLC1 seems to be ubiqutinylated and processed at least partly by ubiquitinylation-dependent proteasomal degradation [117]. Nevertheless, the sites and the mode of ubiquitinylation (mono- or poly-) on the molecule are unclear. In addition, a proinflammatory protein S100A10, a key cell surface receptor for plasminogen and a regulator of proteolysis, was found to be a novel binding partner of START-GAP1/DLC1 [118]. S100A10 colocalizes with START-GAP1/DLC1 in the cytoplasm via interaction between the C-terminus of S100A10 and the central domain of START-GAP1/DLC1, regulating tumor cell invasion [118]. These results strongly suggest that proteolysis dependent on post-tran-scriptional modification of START-GAP1/DLC1 plays an important role in the regulation of its tumor suppressive activity.

7. Possible roles of START-GAP1/DLC1 in the development of vascular diseases

Recently, another potential role of START-GAP1/DLC1 in the pathogenesis of a human disease has emerged. It has been known that the PLC activity with its effect on the regulation of intracellular Ca^{2+} levels has potential roles in cardiac regulation. PLCδ1 activity has shown to be enhanced in patients with coronary artery spasm, CSA (coronary spastic angina) [119, 120]. In this context, a new finding that START-GAP1/DLC1 protein levels and mRNA levels in fibroblasts from Japanese patients with CSA were enhanced compared with levels in control subjects is noteworthy [121]. The authors also found in the START-GAP1/DLC1 promoter analysis, the -228G/A and -1466C/T variants found associated with CSA patients using 5'-RACE method revealed the increase in luciferase activity [121]. The incidence of -228G-A was more frequent in male patients with CSA than in male control subjects, suggesting that this variant is a possible candidate responsible for upregulation of START-GAP1/DLC1 protein in CSA. Thus, it was postulated that START-GAP1/DLC1 is up-regulated in patients with coronary spasm, possibly by mutations in the promoter region, causing increased $[Ca^{2+}]_i$ to acetylcholine, and thereby seems to be related to enhanced coronary vasomotility.

As stated in the earlier section, START-GAP1/DLC1 plays two important roles in signaling pathways: One of the dual functions is the ability to enhance the PtdIns(4,5)P_2-hydrolyzing activity of PLCδ1 and the other is the GAP activity specific for RhoA and Cdc42. The former leads to both the protein kinase C-mediated pathway and Ca^{2+}-dependent pathway, resulting

in enhanced myosin light chain phosphorylation that plays an important role in the constrictor response of the coronary artery smooth muscle to transmitters such as serotonin and histamine. Homma and Emori showed that recombinant PLCδ1 catalyzes the hydrolysis of PtdIns(4,5)P_2 in a Ca^{2+}-dependent manner; in the presence of START-GAP1/DLC1, its activity is 5- to 10-fold increased in the range of physiological Ca^{2+} concentration [22]. Thus, it is conceivable that upregulation of START-GAP1/DLC1 protein observed in patients with CSA is responsible for the high activity of PLCδ1. These results are consistent with the previous finding of the H[257]R variant of PLCδ1, in which the conformational change is associated with upregulation of PLCδ1 activity [120]. These characteristics seem to explain the pathogenesis of CSA in which both the basal vascular tone and the vasoconstrictor response to the diverse stimuli were enhanced. Previously, PLC activity was shown to positively correlate not only with basal coronary artery tone but also with the maximal and averaged constrictor responses of the coronary artery to acetylcholine [119]. This finding also suggests a critical role of START-GAP1/DLC1 protein in the genesis of coronary spasm.

The START-GAP1/DLC1 protein has another function, a GAP activity for Rho GTPases. Alterations in RhoA/ROCK pathway have been implicated in the development of a variety of cardiovascular diseases, including hypertension, atherosclerosis, and cerebral and coronary vasospasm [122, 123].

It was reported that the ROCK inhibitor fasudil attenuated the constrictor response of the coronary artery to ACh and prevented the occurrence of chest pain in patients with CSA [124]. By its GAP activity, START-GAP1/DLC1 may antagonize the development of coronary spasm like the ROCK inhibitor. On the other hand, Ca^{2+} mobilization induced by PLCδ1 activation may upregulate Rho and ROCK in the vascular smooth muscle [125]. Thus, the role of START-GAP1/DLC1 in the regulation of Rho is complicated, and the relation of START-GAP1/DLC1 to the genesis of coronary spasm via Rho activity remains to be determined.

The mechanisms of enhancement of START-GAP1/DLC1 promoter activity by the -228G/A variant may be related to transcription factor SP because this variance causes loss of binding to SP1 in its region [121]. Upregulation of START-GAP1/DLC1 protein in the coronary arteries of CSA patients should be confirmed. It remains unclear whether upregulation of START-GAP2/DLC2 or START-GAP3/DLC3 is associated with CSA.

Regarding the caveola localization of START-GAP1/DLC1, the recent report by Nuno et al. that RhoA colocalization with caveolin-1 in caveolae regulates vascular contractions to serotonin is of interest [126]. Caveolins are not only structural components of caveola micro-domains, but also regulate assembly and activation of a variety of receptors and signaling molecules such as PtdIns(4,5)P_2, glucose transporter 4 (GLUT4), epidermal growth factor receptors (EGFR) and endothelial nitric oxide synthase (eNOS). Localization of RhoA to caveolae versus noncaveolar lipid rafts differentially regulates its activation and contractions to RhoA-dependent agonists with greater activation associated with its localization to noncaveolar rafts. Presumably, translocalization of START-GAP1/DLC1 to caveolae by agonists reported by Yamaga et al. [24, 28] may also contribute to modulate the activity of RhoA/ROCK pathway in vascular cells.
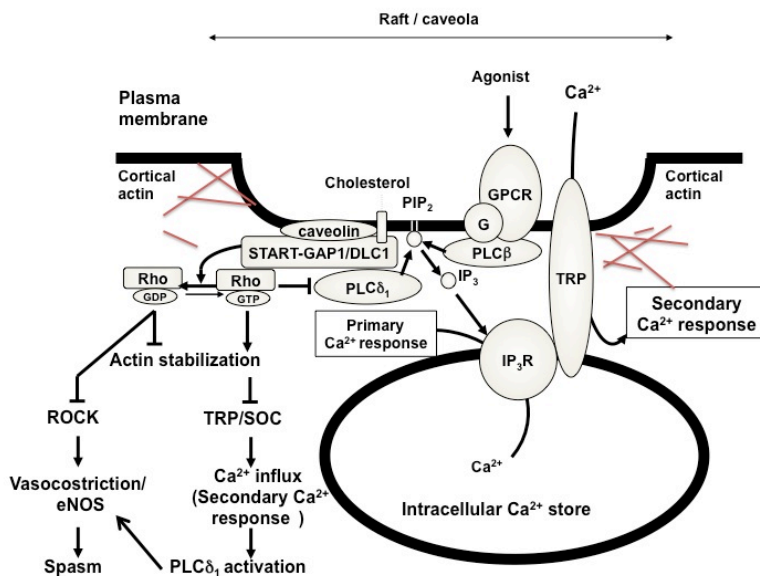
Figure 4. Roles of START-GAP1/DLC1 in a lipid raft/caveola and its possible involvement in the pathogenesis of coronary vasospasm. When G protein coupled receptors (GPCRs) such as muscarinic receptors are activated by agonists they initially stimulate β-type PLC resulting in the production of Ins(1,4,5)P_3 from PtdIns(4,5)P_2. Ins(1,4,5)P_3 binds to the receptors (IP3R or ER Ca^{2+} pump) at the intracellular Ca^{2+} stores such as ER and mobilizes Ca^{2+} from the stores. PLCδ1 is localized at the plasma membrane and in the cytosol in unstimulated cells, whereas START-GAP1/DLC1 is localized in lipid rafts via binding with cholesterol (or with caveolin if the lipid rafts are caveolae). The agonist-induced primary increase in Ca^{2+} recruits PLCδ1 into lipid rafts from other parts of the plasma membrane or from the cytosol by unknown mechanisms. PLCδ1 in the lipid rafts then binds START-GAP1/DLC1 to be activated in the presence of Ca^{2+}, resulting in a robust hydrolysis of PtdIns(4,5)P_2 that forms clusters in lipid rafts. Released Ins(1,4,5)P_3 then empties the internal Ca^{2+} stores via activation of IP3R followed by stimulation of TRP channels, a component of store-operated channels (SOCs) to increase Ca^{2+} influx (secondary Ca^{2+}). Thus the recruitment of PLCδ1 to rafts confers a general positive feedback mechanism for phosphoinositide/Ca^{2+} signaling in the cells. START-GAP1/DLC1 also modulates the RhoA/ROCK signaling pathway. Modified from [24].

8. Conclusion

The START-GAP/DLC family proteins are a group of Rho GTPases whose aberrant function suggests dysregulation of numerous cell processes that can develop diseases such as cancers and vascular spasm. Both in vitro and in vivo studies have provided strong evidence that START-GAP/DLCs have roles in regulating actin cytoskeleton organization. Binding to tensins and other molecules may target the RhoGAP activity of the START-GAP/DLCs to particular subcellular domains. Accumulating reports indicate that the START-GAP/DLC proteins are regulated in various ways at a genetic level by gene deletion, at an epigenetic level by the aberrant promoter methylation, or at a cellular level by the regulation of localization and stability. More information is required on the mechanisms responsible for these regulations,

including post-translational modifications such as phosphorylation and ubiquitination. In addition, dissection of the interacting partners of START-GAP/DLCs by proteomic analyses will reveal detailed signaling pathways that regulate cell shape, motility and proliferation. Future studies on the difference among START-GAP/DLCs may reveal which member of this family plays a key role in individual types of cancer or in vascular abnormality and whether restoration of the proper function of the START-GAP/DLCs is capable of restraining cell transformation or abnormal signaling. These efforts would result in identification of the member that becomes the subject of future studies as a potential drug target for therapy.

Nomenclature

CSA: cardiac spastic angina, DLC: deleted in liver cancer, GAP: GTPase activating protein, HCC: hepatocellular carcinoma, Ins(1,4,5)P_3: inositol 1,4,5-trisphosphate (IP$_3$), NLS: nuclear localization signal, PLC: phosphoinositide-specific phospholipase C, PKB: protein kinase B, PtdIns(4,5)P_2: phosphatidylinositol 4,5-bisphosphate (PIP$_2$), ROCK: Rho-kinase, START: steroidogenic acute regulatory protein (STAR)-related lipid transfer

Acknowledgements

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MIG-6 and SPRY2 in the Regulation of Receptor Tyrosine Kinase Signaling: Balancing Act via Negative Feedback Loops

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Additional information is available at the end of the chapter

1. Introduction

Tumor suppressor genes (TSGs) function in concert with diverse parts of the cellular machinery and integrate the signaling networks in a cell [1]. TSGs act to safeguard the networks, to fine-tune signaling outputs, and to maintain tissue homeostasis. The loss of tumor suppressor activity or inactivation of a TSG is often due to genetic alterations such as a loss-of-function mutation or a deletion in the gene; alternatively, epigenetic silencing can result from methylation or histone modification in the TSG's promoter regulatory elements [1-3]. Cells with a loss or a significant reduction of a particular tumor suppressor's activity are prone to develop neoplasia in the tissues/organs where the TSG is expressed [1, 2].

The properties and modes of action of TSGs can be very distinct from one class to another. Most TSGs encode proteins that participate in controlling cell cycle progression, inducing apoptosis, repairing damaged DNAs, or performing other important functions [1]. TSGs can also be a source of microRNAs, a class of small hairpin RNAs that are transcribed in many cells and may act as tumor suppressors by regulating the expression of their targeted genes [4]. In this chapter, we will focus on one class of the TSGs, represented by the mitogen-inducible gene 6 (MIG-6) and Sprouty 2 (SPRY2), whose activities are crucial in regulating receptor tyrosine kinases signaling through negative feedback loops.

Receptor tyrosine kinases (RTKs) are important cellular components, and there are nearly 60 members encoded in the genome [5, 6]. They all possess a single transmembrane domain linking their extracellular ligand-binding region to the cytoplasmic region in which the catalytic kinase domain and the domain for docking of downstream signaling molecules reside. Upon binding of the ligand to its physiologic RTK partner, the receptors dimerize, resulting

in autophosphorylation of key tyrosine residues in the kinase domain. This leads to a conformational change in the receptor and the recruitment of downstream signaling molecules to its docking domain or in close proximity for phosphorylation and activation. In this cellular process, the RTK plays a central role by relaying external stimuli (ligands) to internal signaling cascades such as the RAS-MAPK or PI3K-AKT pathways, translating the signal input into biological actions ranging from mitogenesis, to motility, morphogenesis, metabolism, and many others [5, 6].

RTK signaling is essential in many developmental processes and in normal physiology, and the actions of RTKs must be controlled temporally and spatially [5, 6]. Their actions are tightly regulated at several molecular levels to ensure appropriate cellular responses. Among the mechanisms that keep RTK signaling in "check and balance" are receptor endocytosis/degradation, dephosphorylation by protein tyrosine phosphatases (PTPs), and negative feedback regulation. Signal overactivity caused by inappropriate RTK activation can lead to serious pathological outcomes, particularly cancer. Thus, many RTKs such as epidermal growth factor receptor (EGFR) and the N-methyl-N'-nitroso-guanidine human osteosarcoma (MNNG HOS) transforming gene (MET) have been classified as oncogenes, because activating mutations, amplifications or other anomalies in these receptors have been identified in various human cancers, and their roles in the development and progression of tumor malignancy have been well documented [7, 8]. For example, aberrant activation of MET can result in deregulated cell proliferation, transformation, and promotion of tumor cell invasion and metastasis [8].

Unlike the rapid attenuation resulting from receptor endocytosis/degradation or PTP-mediated dephosphorylation, negative feedback regulation of RTK signaling by MIG-6 and SPRY2 is a delayed event because it requires *de novo* mRNA and protein syntheses. The expression of both MIG-6 and SPRY2 can be induced by ligands of many RTKs including epidermal growth factor (EGF), hepatocyte growth factor (HGF), and fibroblast growth factor (FGF) [9, 10]. In turn, MIG-6 and SPRY2 exerts their inhibitory activities on RTK signaling by either directly affecting the receptor itself or by modulating the signaling molecules downstream of the RTK. In this review, we will highlight the current understanding of how MIG-6 and SPRY2 regulates RTK signaling via negative feedback loops, and shed lights on why the loss of their tumor suppressor activities may affect RTK signaling in cancer cells, as well as their impact on cancer therapy.

2. The features and functions of MIG-6 and SPRY2

2.1. MIG-6

MIG-6 (also known as *gene 33*, *ERRFI1* or *RALT*) is a unique and immediate early response gene that is not present in relatively simple organisms like *Drosophila* and *C. elegans*. It emerges in the more complex, higher-order species [9, 11], underlying its importance in evolution. It encodes a 58 kDa nonkinase protein that resides in the cytoplasm and functions as a scaffolding adaptor for modulating signal transduction. Struc-

turally, MIG-6 has several functional motifs/domains that are crucial for interaction with other signaling molecules [9, 12]. The Cdc42/Rac-interaction and binding (CRIB) domain of MIG-6 (Figure 1A) shares consensus sequences with many other proteins that associate with Cdc42 or Rac small GTPases, which are important regulators of actin cytoskeleton remodeling and signal transduction [11, 13]. CRIB domain mediates the binding of MIG-6 to active (GTP-bound) Cdc42 and negatively regulates HGF-induced Cdc42 activation and cell migration [12, 14]. This domain has also been shown to play a role in regulating transactivation of nuclear factor κB (NFκB) by sequestering the inhibitor of κBα (IκBα) [15, 16]. Within its middle region, MIG-6 has several proline-rich motifs that likely mediate its binding to various Src homology-3 (SH3) domain-containing proteins such as GRB2, Src, PI3K p85 and PLC-γ [14, 17]. MIG-6 interacts with 14-3-3ζ via the 14-3-3 protein binding motif [12]. MIG-6 also possesses two PEST sequences, and is targeted by ubiquitination and proteasome degradation [18]. The ErbB-binding region (EBR), a large portion of the carboxyl terminus in MIG-6, is required for physical interaction with EGFR family receptors, which resulted in attenuation of EGFR/ErbBs signaling [17, 19, 20]. The EBR domain shares a high homology with the activated Cdc42-associated kinase 1 (ACK1), a non-receptor tyrosine kinase that also interacts with and regulates EGFR [21].
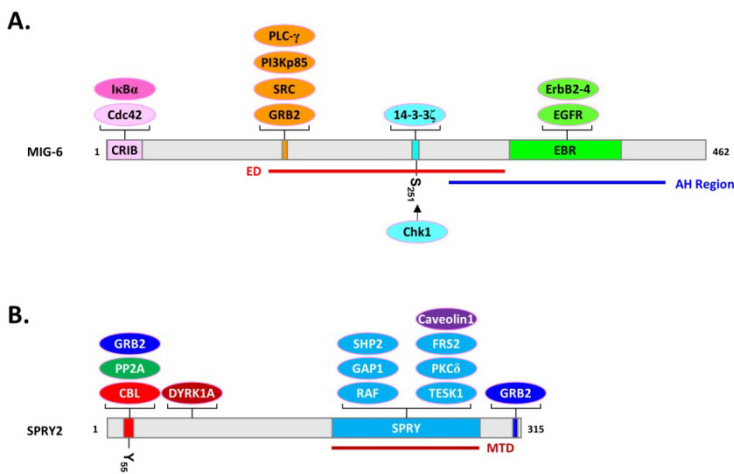
Figure 1. MIG-6 and SPRY2 structures. A. MIG-6 protein structural features and its interacting partners. The CRIB domain (amino acids 1-38) interacts with Cdc42 and IκBα. The orange box indicates the SH3-domain binding motif that likely mediates interactions with SH3-domain-containing proteins such as GRB2 and PI3Kp85. The cyan box (amino acids 246-253) marks the 14-3-3 binding motif in which serine residue 251 can be phosphorylated by Chk1 kinase. The EBR domain (amino acids 337-412) binds to EGFR and other ErbB members. The red bar (ED) indicates the MIG-6 endocytic domain (amino acids 143-323); the blue bar indicates the Ack homology (AH) region (amino acids 264-424). B. Structural features of SPRY2 and its partner molecules. The red box shows the SH2-domain binding motif (amino acids 50-60) that binds to CBL and GRB2; the key tyrosine residue Y_{55} is also indicated. The conserved cysteine-rich SPRY domain (amino acids 178-293) is crucial for its ability to interact with signaling molecules such as FRS2 and SHP2. The SPRY domain is also responsible for membrane translocation (MTD). There is an SH3-domain binding motif (amino acids 303-309) shown in blue at the C-terminal end of SPRY2 that also binds to GRB2.

MIG-6 is expressed in many tissues/organs, with high expression in liver and kidney and low to moderate expression in brain, lung, heart, and other tissues [22, 23]. Its expression can be induced by diverse factors ranging from hormones and growth factors, to chemical agents, to stress stimuli [9]. The induction of MIG-6 expression by growth factors is mainly mediated by the RAS-MEK-MAPK/ERK pathway, while other inducers may involve other pathways such as PI3K [9]. MIG-6 has also been reported to be a G-actin-regulated target gene, because the actin-MAL-serum response factor (SRF) cascade mediates MIG-6 induction by serum or lipid agonists such as lysophosphatidic acid (LPA) or sphingosine 1-phosphate (S1P) [24]. MIG-6 may play a crucial role in patho-physiological conditions such as myocardial ischemic injury and infarction [25], liver regeneration [26-28], joint mechanical injury [29], and diabetic nephropathy and hypertension [12]. Its activity is required for skin morphogenesis [30, 31] and lung development in mice [32], and it plays an important role in the maintenance of tissue homeostasis in joints, the lungs and the uterus [32-35].

2.2. SPRY2

In term of evolution, the *Spry* gene emerged far earlier than *Mig-6*. *SPRY2* is the mammalian homolog of *Drosophila melanogaster Spry* (*dSpry*), and is one of the four *SPRY* genes in the human genome [10]. The dSpry protein is 63 kDa, while its mammalian counterparts are 32–34 kDa, but they all contain a functional cysteine-rich region in their C-terminus (designated the SPRY domain) and an SH2-binding motif carrying a conserved tyrosine at the N-terminus (Tyr55 residue in human SPRY2) (Figure 1B). These conserved regions are essential for SPRY2 to fully execute its inhibitory function in the regulation of RTK signaling [10, 36, 37]. The SPRY domain is also found in the SPRED (Sprouty-related EVH1 domain-containing protein) family, which like SPRY proteins inhibits RTK signaling upon stimulation by growth factors [10, 38]. The SPRY domain mediates the binding of SPRY2 to many signaling molecules including GAP1, FRS2, SHP2, RAF, PKCδ, TESK1 and caveolin1 [10, 39]. The SPRY domain is also required for translocation of SPRY2 to the membrane during its activation. The Tyr55 residue is essential for the SPRY2 protein's interaction with CBL, PP2A and GRB2 [10, 39]. A cryptic SH3-binding motif (PxxPxR) in the C-terminal end of SPRY2 (but not present in other SPRY members) has been shown to mediate GRB2 interaction as well [39-41]. The dual-specificity tyrosine phosphorylation–regulated kinase (DYRK1A) interacts with and phosphorylates Thr75 on SPRY2 [42]. SPRY2 is targeted for ubiquitination and proteasome degradation by at least two ubiquitin E3 ligases: CBL-mediated Tyr55 phosphorylation-dependent ubiquitination and SIAH2-mediated Tyr55 phosphorylation-independent ubiquitination [43, 44]. On the other hand, SPRY2 protein can be stabilized by phosphorylation of its serines 112 and 121 residues by the mitogen-activated protein kinase-interacting kinase 1 (Mnk1), thereby decreasing growth factor–induced degradation [45].

In *Drosophila*, *dSpry* expression is detected at the tips of branching lung buds and is induced by branchless, the *Drosophila* Fgf. Losing *dSpry* leads to excessive tracheal branching as a result of increased Fgf signaling activity [46]. The *Xenopus* homolog, *xSpry2*, is expressed in a pattern resembling that of *Xenopus* Fgf8 and inhibits Fgf-mediated gastrulation [47]. During mammalian embryogenesis, Spry2 expression tends to localize closest to the sites where Fgf

activity is needed for organ/tissue development, underlying the importance of this molecule as an intrinsic Fgf signaling regulator in organogenesis [48-50]. Mouse *Spry2* is highly expressed in the terminal buds of peripheral mesenchyme in the embryonic lung, adjacent to that of Fgf10, a key mouse lung-branching morphogen [50]. Ectopic expression of Spry2 in the mouse pulmonary epithelium results in decreased branching; exogenous Fgf10 produces greater lung branching and higher Spry2 expression [50]. Spry2 also plays a role in mouse kidney development; its ectopic expression in the ureteric bud leads to postnatal kidney failure due to deficiency in ureteric branching [51]. Moreover, Spry2 deficiency in mice results in defects of the auditory sensory epithelium development in the inner ear, and leads to enteric neuronal hyperplasia and esophageal achalasia [52, 53]. The lack of phenotypes in other Spry2-deficient tissues is likely due to compensatory roles of other Spry family members, because there are overlapping expressions of Spry1, 2 and 4 in many tissues during the development [54]. In adult mice, Spry2 expression is abundant in the brain, lung, heart, kidney, skeletal muscle and mammary glands [48, 55].

Beyond being an intrinsic inhibitor for Fgf signaling, Spry also regulates signaling driven by other RTKs like Egfr. The *dSpry* gene is required for eye and wing development in *Drosophila*, antagonizing Egfr signaling for neuronal induction in the retina and for vein formation in the wings [56, 57]. Loss of *dSpry* results in excess photoreceptors, cone cells and pigment cells in the retina, while its overexpression leads to phenotypes that mimic loss of Egfr signaling [56, 57].

3. Negative feedback regulation of RTK signaling by MIG-6 and SPRY2

3.1. Regulation of RTK pathways by MIG-6

Many growth factors can induce MIG-6 expression, including EGF, FGF, and HGF [9]. Upon induction, MIG-6 proteins rapidly and transiently accumulate in the cytoplasm, where they feed back to inhibit the activated RTK signaling (Figure 2). The inhibition of EGFR/ErbB signaling by MIG-6 can occur at two molecular levels: one on the receptor itself, and another on the signaling molecules downstream of the receptor (Figure 2). Through its C-terminal EBR domain, MIG-6 directly binds to EGFR and other ErbB members [17, 19, 20, 58]. The interaction involves the kinase domain of EGFR or ErbB2 and requires their catalytic activities, but does not involve their C-terminal regions in which there are tyrosine residues essential for activating downstream signaling [20, 58]. Crystal structures reveal that the MIG-6 EBR domain binds to the distal surface of the carboxy-terminal lobe (C-lobe) in the kinase domain of EGFR [59]. The C-lobe is crucial in asymmetric EGFR dimer formation [60]; binding of MIG-6 to the C-lobe blocks the dimer interface thereby preventing EGFR activation [59]. MIG-6 coupling also promotes clathrin-mediated endocytosis of EGFR [61], an important mechanism for timely attenuation of ligand-induced EGFR activation [62, 63]. The region responsible for promoting EGFR endocytosis has been mapped to the endocytic domain (ED) of MIG-6 (see Figure 1A) [61], which mediates the binding of MIG-6 to the AP2 adaptor complex, a key component in forming clathrin-coated pits during endocytosis [63]. Interest-

ingly, the non-receptor tyrosine kinase ACK1 also binds to EGFR upon EGF stimulation through a region sharing high homology with the MIG-6 EBR domain [21]. This interaction also regulates clathrin-mediated EGFR endocytosis and degradation [21, 64]. However, it is not clear whether MIG-6 and ACK1 cooperate in regulating EGFR turnover or whether they bind to EGFR in a mutually exclusive way to accomplish individual inhibitory roles under different circumstances. The internalized EGFR is guided to late endosome through the binding of MIG-6 to the endosomal SNARE complex component STX8, en route to degradation in the lysosome [61, 65].
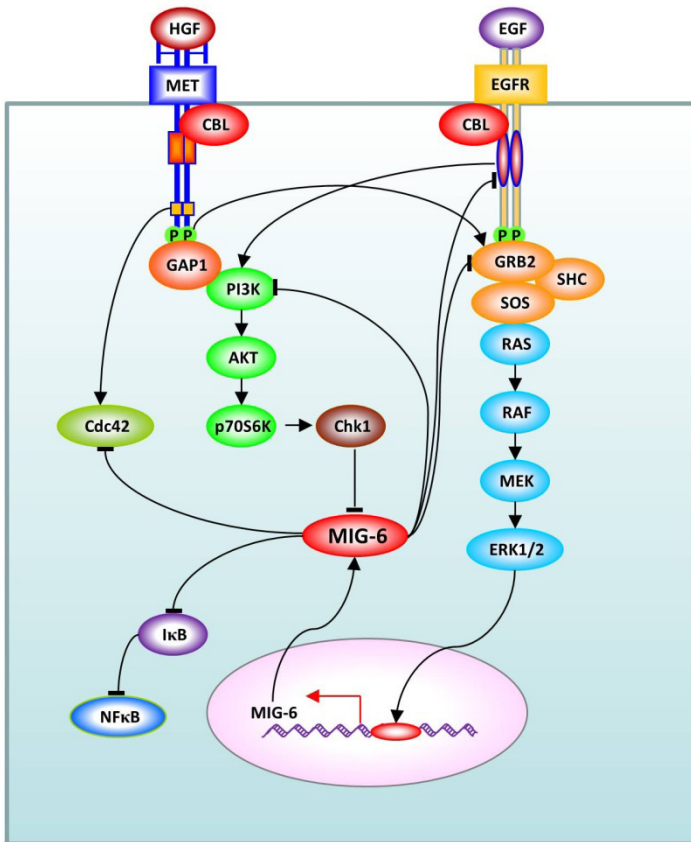
Figure 2. MIG-6 regulates EGFR and MET signaling via a negative feedback loop. Upon ligand stimulation, EGFR and MET activate the RAS-RAF-MEK-ERK pathway and induce *MIG-6* expression. In turn, MIG-6 exerts its inhibitory activity by interacting with signaling molecules to fine-tune RTK signaling and its timely attenuation. The direct MIG-6–EGFR interaction facilitates receptor endocytosis and degradation. This inhibitory activity is unique to the EGFR family and does not extend to other RTKs like MET. The interaction of MIG-6 with signaling molecules such as GRB2 and PI3Kp85 indistinguishably influences the RTK signaling in general, resulting in the inhibition of downstream pathways like RAS-MAPK/ERK and PI3K-AKT.

The magnitude of MIG-6-mediated inhibition of EGFR signaling is likely maximized and integrated by the second level of molecular regulation, that is, direct inhibition of downstream signaling molecules (Figure 2). MIG-6 binds to the SH3 domain-containing protein GRB2 [14, 17], the key molecule in linking activated RTK to the intracellular signaling cascade; GRB2 brings RAS together with SOS to the phosphorylated receptor for activation [66]. Binding of MIG-6 may block GRB2's interaction with activated EGFR and other RTKs or disrupt GRB2-SOS-RAS complex formation, thereby preventing activation of the RAS-MEK-MAPK pathway. MIG-6 also interacts with Src, the PI3K p85 subunit, PLCγ, and Fyn [17], although those interactions were demonstrated in an artificial system and their biological meaning remains to be determined. More complexity is likely in the dynamics of MIG-6-mediated RTK signaling regulation due to the interaction of MIG-6 with 14-3-3 proteins [12, 67], an adaptor family that may interact with diverse signaling molecules and regulate many biological activities [68]. Nonetheless, most of these direct downstream signaling regulations by MIG-6 remain largely speculation and require further investigation.

Assessing the effect of MIG-6 on RTK pathways other than those of the EGFR family, however, may provide insightful answers to such speculation, because direct regulation of the RTK itself by MIG-6 appears to be unique to the EGFR family. For instance, MIG-6 can be induced by HGF and function as negative feedback regulator of the MET pathway by inhibiting HGF-induced cell migration and proliferation [14], yet no physical interaction between MIG-6 and MET has been observed. Through its CRIB domain, MIG-6 can bind to and inhibit the activity of the Cdc42 small GTPase [12, 14], and this inhibitory activity is required for blocking of HGF-induced cell migration [14]. The CRIB domain also interacts with IκBα, thereby activating NFκB for transcriptional regulation of its target gene expression [15, 16]. Whether the inhibition of HGF-induced cell proliferation by MIG-6 is mediated by its ability to bind GRB2 or to bind other downstream molecules is still unknown. Negative feedback inhibition of other RTKs (such as FGFR and IGFR) by MIG-6 is also likely to be mediated by its inhibitory activities on the downstream signaling molecules rather than on the receptor itself.

The inhibitory activity of MIG-6 on RTK signaling seems to be modulated by phosphorylation; Liu et al. recently reported that MIG-6 can be phosphorylated by Chk1 upon EGF stimulation [67]. EGF activates Chk1 via the PI3K-AKT-S6K pathway, which in turn phosphorylates Ser251 of MIG-6 and results in inhibition of MIG-6 [67]. Thus, Chk1 counterbalances the EGFR inhibition of MIG-6, positively regulating EGFR signaling. Interestingly, Ser251 resides in the 14-3-3 binding motif of MIG-6 and its phosphorylation is likely involved in the MIG-6 and 14-3-3ζ interaction, because that interaction is abolished by Chk1 depletion [67]. In addition, two tyrosine residues (Tyr394 and Tyr458) in MIG-6 are phosphorylated by EGFR activation [69-71], but the underlying mechanism and the biological significance remain unclear.

3.2. Regulation of RTK pathways by SPRY2

SPRY2 renders another layer of modulation on RTK signaling via a negative feedback loop [10, 36, 37, 72]; its expression is induced by many activated RTKs including EGFR, FGFR,

MET, and VEGFR [10, 72]. As with MIG-6, SPRY2-mediated regulation can occur on two levels: on the RTK itself and on the downstream signaling molecules (Figure 3). However unlike MIG-6, the most prominent inhibitory activity of SPRY2 is derived from its abilities to interact with downstream signaling molecules centering on the RAS-RAF-MAPK pathway, while its effect on the RTK itself seems to be indirect and may provide some signaling specificity for different RTKs [10, 36, 37].
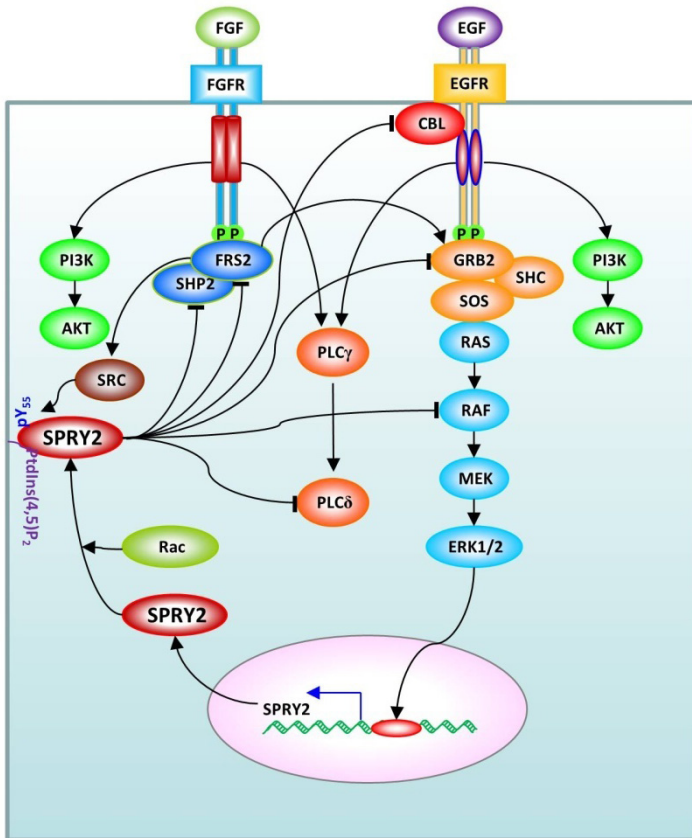
Figure 3. Feedback regulation of EGFR and FGFR signaling by SPRY2. SPRY2, upon induction, translocates to the membrane by binding to PtdIns(4,5)P_2, and is phosphorylated on its Y_{55} residue. This modification is essential for SPRY2's inhibitory activity, which is mediated by interaction with many signaling proteins including FRS2, SHP2, GRB2, RAF and PLCδ. On the other hand, its interaction with CBL E3 ubiquitin ligase may positively regulate EGFR signaling, because such interaction sequesters CBL and prevents CBL-mediated EGFR degradation.

Upon growth factor stimulation, SPRY2 translocates from the cytosol to the plasma membrane where it binds to phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P2] via the conserved SPRY domain [73-75]. Membrane translocation appears to be triggered by the activated Rac small GTPase and is essential for SPRY2 function [76]. The phosphorylation of the Tyr55 residue in SPRY2 is likely regulated by a SRC family kinase, which upon FGF stimulation is recruited to activated FGFR by FRS2 [77]. While Tyr55 phosphorylation enables the binding of SPRY2 to GRB2 via the SH2-binding motif in the N-terminus [78], the SH3-binding motif in its C-terminus may also play a role in the SPRY2-GRB2 interaction [39-41] (Figure 1B). The latter is likely regulated by the protein phosphatase 2A (PP2A), a serine/threonine phosphatase that interacts with Tyr55 and dephosphorylates certain serine residues in SPRY2 for permitting access of GRB2 to the SH3-binding motif on SPRY2 [41, 45]. Consequently, the binding of SPRY2 to GRB2 prevents the recruitment of the GRB2-SOS complex to the FRS2 adaptor or SHP2 phosphatase proximal to the activated RTK, thereby inhibiting the activation of RAS and its downstream molecules [10, 36, 39, 77, 78]. The RTK-RAS-RAF-MAPK/ERK pathway can also be inhibited by direct binding of SPRY2 to RAF [75, 79, 80]. On the other hand, TESK1 negatively regulates SPRY2 inhibitory activity by interfering with the SPRY2 interaction with GRB2 and PP2A [81], while DYRK1A binding results in Thr75 phosphorylation on SPRY2 and suppression of SPRY2 inhibitory activity, thereby enhancing FGF-induced ERK activation [42].

The regulation of EGFR signaling by SPRY2 appears to be more complicated than that of FGFR signaling, because SPRY2 can indirectly influence the turnover of EGFR through its interaction with CBL (Figure 3) [10, 36, 37]. This regulation is mediated by the SH2-binding motif, which includes Tyr55 in the N-terminus of SPRY2. When Tyr55 is phosphorylated, SPRY2 interacts with the SH2-domain on CBL, and prevents CBL from binding to the activated EGFR, thereby interfering with CBL-mediated EGFR endocytosis and degradation [43, 82-84]. This action can prolong the activation of EGFR and the downstream RAS-RAF-MAPK pathway, contrary to the direct inhibitory activity of SPRY2 on the downstream signaling molecules. These two opposite activities render SPRY2 a delicate role in fine-tuning EGFR signaling, negative in some situations and positive in others, depending on the threshold and balance of these two activities.

It is conceivable that RTKs such as MET and PDGFR that are also substrates of CBL might also be affected by SPRY2 like that of EGFR [8, 85], while non-CBL-substrate RTKs like FGFR appears unaffected by SPRY2-CBL interaction [36]. However, it is known that MET protein level is not affected by SPRY2 overexpression that inhibits HGF-induced ERK and AKT activation, indicating that the effect of SPRY2-CBL interaction on EGFR and on MET might not be the same [72, 86]. The sequestration of CBL by SPRY2 can also affect downstream signaling molecules, as it may free proteins like GRB2 from CBL-mediated ubiquitination and degradation [85]. Furthermore, SPRY2 itself can be ubiquitinylated and degraded by the CBL binding, thereby influencing the RTK signaling [83].

4. Tumor suppressor role of MIG-6 and SPRY2 in cancer

4.1. MIG-6 as a tumor suppressor gene

Human chromosome 1p36, a locus frequently associated with many human cancers [87-92], harbors the *MIG-6* gene. In fact, loss or reduction of *MIG-6* expression has been observed in non-small cell lung cancer (NSCLC) [35, 93-95], breast carcinoma [30, 96], melanoma and skin cancer [30, 94], ovarian carcinoma [30], pancreatic cancer [30], endometrial cancer [34], thyroid cancer [97, 98], hepatocellular carcinoma [28], and glioblastoma [91]. Prognostically, low *MIG-6* expression is often associated with poor prognosis or poor patient survival [93, 96, 97].

Unlike many other tumor suppressor genes whose expression is directly regulated by epigenetic modification of their promoter regulatory elements [3], the silencing of *MIG-6* expression seems otherwise [94]. Although high in CpG contents, the *MIG-6* promoter appears hypomethylated and is not directly affected by either the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-aza-dC) or the histone deacetylase inhibitor trichostatin A (TSA), indicating indirect regulation [94]. Interestingly, *MIG-6* expression seems to be differently induced by 5-aza-dC and TSA in different cancer types: it is induced by 5-aza-dC in melanoma but not in NSCLC and neuroblastoma, and by TSA in NSCLC and neuroblastoma but not in melanoma, suggesting a possible tissue-specific transcriptional regulation for this gene [90, 94, 96]. However in some papillary thyroid cancer, it is reported that the *MIG-6* promoter is hypermethylated as determined by methylation-specific PCR [98]. It is unclear at this point what cause such differences.

Besides loss or reduction of expression, *MIG-6* can also be inactivated by genetic mutation, even though this occurs rarely [35, 90, 96]. To date, two homozygous mutations (Asp109 to Asn, and Glu83 to a stop codon) in *MIG-6* were identified in human lung cancer cell lines, while heterozygous germline mutations were found in a primary lung cancer (Ala373 to Val) and a neuroblastoma patient (Asn343 to Ser) [35, 90]. Evidence that *MIG-6* is a bona fide tumor suppressor gene also arises from mouse studies. Mice with targeted disruption of *Mig-6* are prone to neoplastic development ranging from epithelial hyperplasia to carcinoma at multiple sites including the lung, gallbladder, bile duct, uterus, gastrointestinal tract and skin [30, 34, 35]. The carcinogen-induced skin cancer seen in Mig-6-deficient mice is likely mediated by EGFR-ERK/MAPK pathway, because inhibiting EGFR kinase activity with gefitinib or replacing it with a kinase-defective EGFR rescues the phenotype [30].

4.2. SPRY2 as a tumor suppressor gene

There is compelling evidence supporting SPRY2 as a tumor suppressor [99]. The *SPRY2* gene is located on human chromosome 13q31, where loss of heterozygosity (LOH) is observed in prostate cancer and hepatocellular carcinoma [86, 100]. Down-regulation of *SPRY2* expression has been reported in breast cancer [55, 101], hepatocellular carcinoma [86, 102, 103], NSCLC [104], prostate cancer [100, 105, 106], endometrial carcinoma [107], gliomas [108], and B-cell lymphomas [109, 110]. However in colon cancer, both downregulation and

upregulation of *SPRY2* have been reported [111, 112]. Low (or no) *SPRY2* expression is asso-ciated with advanced tumor stages and poor survival, and it may be a significant prognostic factor in breast cancer [101], hepatocellular carcinoma [86, 103], prostate cancer [100, 105], gliomas [108], and colon cancer [111]. Further, *SPRY2* expression is inversely correlated with the level of miR-21 microRNA expression in gliomas and colon cancer, indicating that *SPRY2* is a target of miR-21[108, 111].

Downregulation of SPRY2 expression may be attributed to DNA methylation; hypermethy-lation of its promoter has been observed in prostate cancer [100], hepatocellular carcinoma [86], endometrial carcinoma [107] and B-cell lymphomas [109, 110]. However, controversial results have also been reported, since no methylation in *SPRY2* promoter was found in other studies involving different cancer types [55, 102, 106, 111], indicating that other epigenetic mechanisms might as well be responsible for *SPRY2* down-regulation. Thus far, no mutation has been identified in the *SPRY2* gene in any human cancers, and no neoplastic phenotypes have been observed in any Spry2-deficient mice. This may be due to compensatory roles played by other family members such as SPRY1 or SPRY4.

5. The impacts of MIG-6 or SPRY2 activity on RTK signaling in cancer

The loss of MIG-6 and SPRY2 feedback regulation leads to prolonged RTK signaling activa-tion and may contribute to hallmark activities of cancer [113]. Ectopic expression of MIG-6 results in decreased phosphorylation of EGFR/ErbBs and downstream ERK/MAPK and AKT, and it inhibits cell proliferation in several cancer types [19, 20, 65, 96, 114]. In contrast, down-regulation of *MIG-6* expression by small interference RNA (siRNA)-mediated knock-down leads to prolonged activation of the EGFR or ErbB2 pathway and increases ligand-in-duced proliferation, cell cycle progression, and cell migration [28, 30, 65, 96, 114]. Likewise, *MIG-6* overexpression inhibits HGF-induced cell migration and proliferation, whereas MIG-6 knockdown by siRNA enhances those activities [14]. Intriguingly, it has been shown that in thyroid cancer, *MIG-6* overexpression suppresses MET phosphorylation along with the inhibition of EGFR, ErbB2, and SRC, while its knockdown does the opposite and enhan-ces cell proliferation and invasion [98]. However, it is unclear how MIG-6 affects MET tyro-sine phosphorylation given that no physical interaction between the two is observed [14]. The activity of MIG-6 on apoptosis is unsettled: one group showed that MIG-6 inhibits apoptosis of breast cancer cells [115], while another showed that it promotes the death of cardiomyocytes [25]. This discrepancy might be due to the differences in cellular states (can-cer cells versus normal cells) or in tissue types (breast versus cardiac).

Nonetheless, a study of NCI-60 cell lines, which cover a broad spectrum of cancer types, re-vealed that *MIG-6* expression correlated with EGFR expression, indicating an intrinsic activ-ity for MIG-6 in regulating EGFR signaling [116]. *MIG-6* expression has been shown to have a significant effect on ErbB-targeted cancer therapy [96, 116, 117]. MIG-6 synergizes with the EGFR inhibitor gefitinib to suppress the growth of NSCLC cells carrying gefitinib-sensitive EGFR mutations [116, 117]. Further, the loss of *MIG-6* expression renders ErbB2-amplified

breast cancer cells more resistant to Herceptin (trastuzumab), a neutralizing antibody against ErbB2/HER2 [96]. It will be interesting to see the influence of *MIG-6* expression on cancer therapies targeting other RTKs such as MET as well.

SPRY2 overexpression inhibits MET-mediated ERK and AKT activation in leiomyosarcoma and hepatocellular carcinoma cells, and it suppresses cell proliferation, migration and invasion induced by HGF [72, 86, 102]. In NSCLC, SPRY2 suppresses ERK but not AKT activation, and it inhibits the migration of the cells expressing wild-type but not constitutively activated K-RAS; however, proliferation and tumorigenesis of cells with either wild-type or mutant K-RAS can be inhibited by *SPRY2* overexpression [104]. SPRY2 has different effects on wild-type and V599E mutant B-RAF in melanoma cells: its downregulation enhances ERK phosphorylation only in melanoma cells carrying wild-type B-RAF, likely because of its ability to interact with wild-type, but not mutant, B-RAF [118]. Overexpression of *SPRY2* suppresses ERK activation in osteosarcoma and B-cell lymphoma, and it inhibits tumor growth and metastasis *in vivo* [109, 119], while suppression of SPRY2 activity by its dominant negative mutant SPRY2^{Y55F} enhances the proliferation and tumorigenesis of breast cancer cells [55]. Surprisingly, SPRY2 has also been reported to enhance cell proliferation and HGF-induced ERK and AKT activation, migration and invasion of colon cancer cells [112], quite opposite to another report showing that in the same cell line, SPRY2 negatively regulates ERK and AKT phosphorylation and inhibits proliferation, migration and tumorigenesis [111]. The discrepancy is quite puzzling, and it is unclear whether it is due to differences in the experimental approaches or to other factors such as clonal effects originating from tumor cell heterogeneity. Using an inducible system in the same cell line might be able to solve the puzzle of whether the effects in those two reports were truly the results of *SPRY2* overexpression.

There is limited evidence that *SPRY2* expression, like that of *MIG-6*, influences ErbB-targeted therapy in human cancers [101, 120]. Breast cancer patients with low *SPRY2* expression show poorer response to trastuzumab treatment, and have a significant lower survival rate relative to those with high *SPRY2* expression [101]. Low *SPRY2* expression is usually associated with high ErbB2/HER2 in breast cancer, while reconstituting SPRY2 may enhance the sensitivity of breast cancer cells *in vitro* to trastuzumab treatment [101]. In colon cancer cells, low *SPRY2* expression is associated with less sensitivity to gefitinib, whereas its ectopic overexpression can enhance gefitinib responsiveness [120].

6. Conclusion and perspective

The negative feedback loops to receptor tyrosine kinases of MIG-6 and of SPRY2 provide crucial intersecting points for tumor suppressor genes and oncogenes, placing them in the same signaling networks for regulating physiologic and oncogenic activity. This sophisticated regulatory mechanism allows timely attenuation of RTK signaling by those TSGs, and ensures a proper cellular response following growth factor stimulation. MIG-6 and SPRY2 are no more than the representatives for the class of TSGs involved in RTK

signaling regulation, and we believe there are more such TSGs in the human genome either remain to be discovered or have already been revealed (such as other SPRY family members). To date, most of the studies on MIG-6 and SPRY2 have focused on their activity in regulating selected RTKs such as EGFR, MET and FGFR. Their roles in regulating most other RTKs and the clinical relevance of such regulation remain largely unknown. Also, there are conflicting results on how MIG-6 and SPRY2 may regulate the RTK signaling, on both the receptor and the downstream signaling levels, and further studies are required to address those issues. Although EGFR and other RTKs like MET appear to be regulated slightly differently by MIG-6 and SPRY2, it remains to be determined to what extent these TSGs may provide signaling specificity to different RTKs. Beyond all aforementioned issues, a broader question might be why an RTK network needs multiple negative feedback regulators to fine-tune its signaling; might the regulators function differently for each RTK in a temporal and spatial manner i.e. at right place, on right time and for right target?

While conventional mechanisms such as mutation or promoter methylation may contribute to the inactivation of MIG-6 or SPRY2 tumor suppressor roles, their activities are also likely silenced by unconventional means in cancer. For example, in most cancer types investigated so far, *MIG-6* expression is not down-regulated by direct methylation or histone deacetylation in its promoter, but rather by an indirect mechanism involving other unidentified transcriptional factor(s) or transcriptional co-regulator(s). It is also striking to see that different promoter methylation status in *MIG-6* or *SPRY2* gene is observed in different cancer types: methylated in some cancers, but unmethylated in others. The cause of such difference is a curiosity, but if only for TSG down-modulation, genome instability in the cancer cell environment could provide many mechanisms.

Clinically, it is important to understand how tumor suppressor genes may affect the therapeutic outcome of RTK-targeted therapies, which can be effective in treating certain human cancers. In a limited number of studies, low expression of *MIG-6* or *SPRY2* is associated with poorer patient responses to ErbB-targeted therapies (i.e., the EGFR inhibitor gefitinib and the ErbB2/HER2 inhibitor Herceptin). The question is, can *MIG-6* or *SPRY2* expression be used in conjunction with RTK status to select patients for RTK-targeted "personalized" cancer therapy? The approach sounds plausible, given that those TSGs negatively regulate the RTK signaling activities, but extensive studies will certainly be required before implementing such measures.

Acknowledgements

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