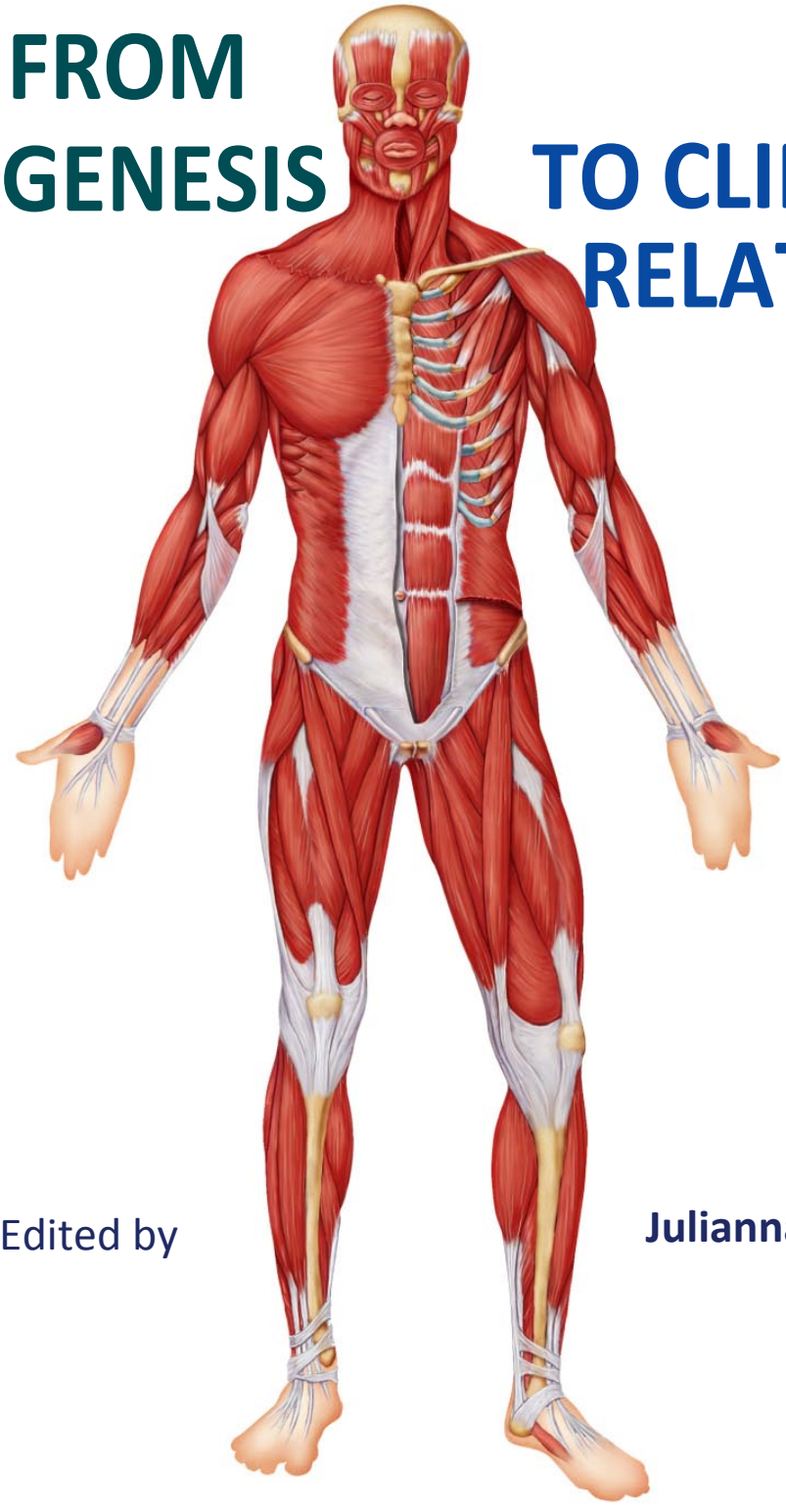
SKELETAL MUSCLE

FROM MYOGENESIS TO CLINICAL RELATIONS



Edited by

Julianna Cseri

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Contents

Preface

The skeletal muscle as an element of the movement system and as a highly differentiated tissue is in the focus of the current scientific investigations. The *in vivo* and *in vitro* development of the muscle cells can be taken as a model for the cell proliferation and differentiation. Ever-increasing knowledge about the mechanism and determinants of the development and the regeneration makes the topic significant both in the theoretical and clinical aspects. Understanding the molecular mechanism of the muscular disorders offers more accurate basis for the therapeutic interventions leading to more effective treatment. The main advantage of this collection is the multidisciplinary approach of the topic. The chapters can be interested for clinicians, medical and health care students as well as researchers in the field of the molecular biology.

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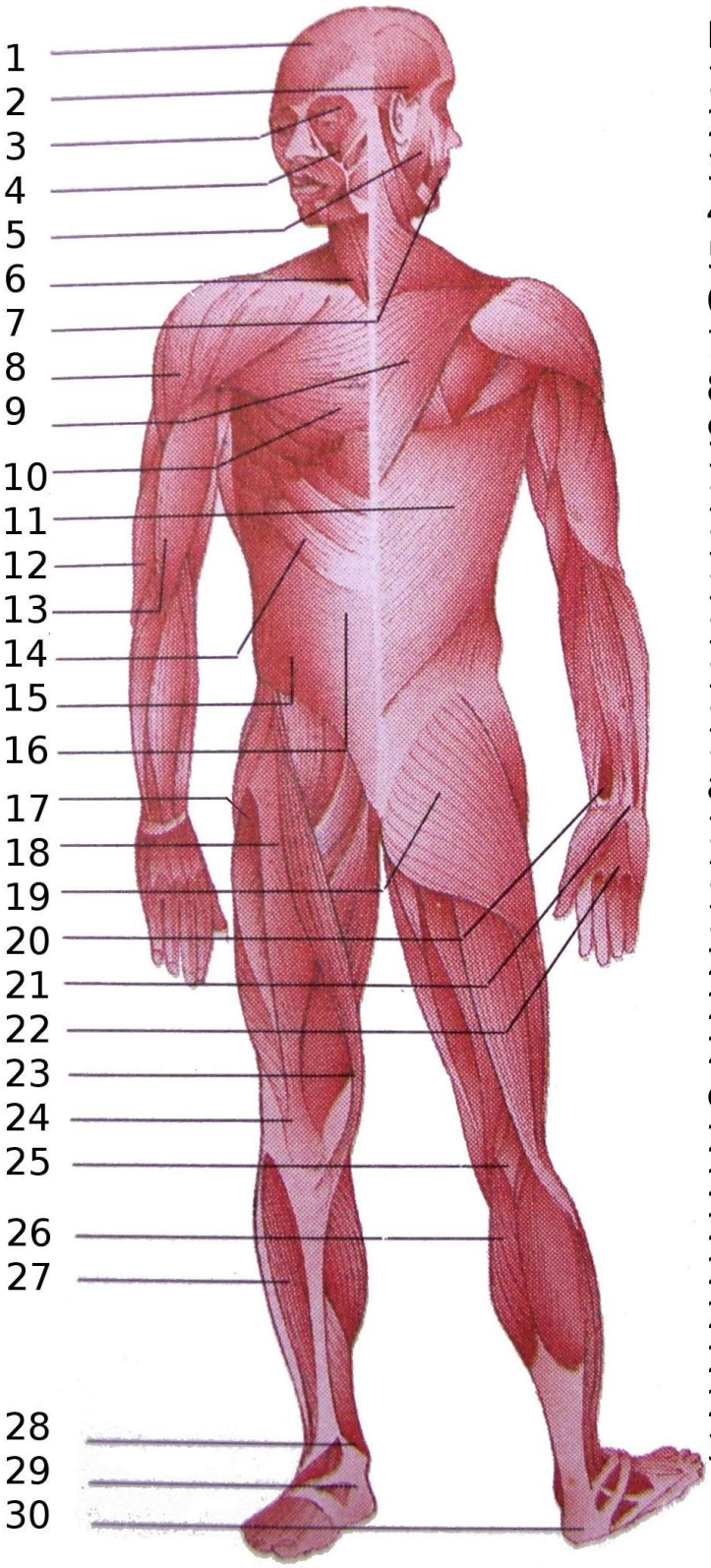
Skeletal muscles



Musculus ...
1:occipitofrontalis
2:temporoparientalis
3:orbicularis oculi
4:levator labii superior
5:masticatorii
6:sternocleidomastoideus
7:orbicularis oris
8:deltoideus
9:trapezius
10:pectoralis major
11:latissimus dorsi
12:triceps brachii
13:biceps brachii
14:serratus anterior
15:rectus abdominis
16:obliquus externus abdominis
17:tensor fascia lata
18:rectus femoris
19:gluteus maximus
20:pronator quadratus
21:flexor retinaculum
22:flexor digitorum communis
23:sartorius
24:quadriceps femoris
25:ischiocrurale
26:gastrocnemius
27:tibialis anterior
28:soleus
29:extensor retinaculum
30:triceps surae

Section 1

Myogenesis and Muscle Regeneration

Chapter 1

Molecular and Cellular Mechanism of Muscle Regeneration

Kunihiro Sakuma and Akihiko Yamaguchi

Additional information is available at the end of the chapter

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1. Introduction

Skeletal muscle contractions power human body movements and are essential for maintaining stability. Skeletal muscle tissue accounts for almost half of the human body mass and, in addition to its power-generating role, is a crucial factor in maintaining homeostasis. Given its central role in human mobility and metabolic function, any deterioration in the contractile, material, and metabolic properties of skeletal muscle has an extremely important effect on human health.

Several possible mechanisms for age-related muscle atrophy have been described; however the precise contribution of each is unknown. Age-related muscle loss is a result of reductions in the size and number of muscle fibers [1] possibly due to a multi-factoral process that involves physical activity, nutritional intake, oxidative stress, and hormonal changes [2-4]. The specific contribution of each of these factors is unknown but there is emerging evidence that the disruption of several positive regulators [Akt and serum response factor (SRF)] of muscle hypertrophy with age is an important feature in the progression of sarcopenia [5-7]. In addition, sarcopenia seems to include the defect of muscle regeneration probably due to the repetitive muscular damage. Indeed, the group of Conboy [8-10] indicates that Notch-dependent signaling is impaired in sarcopenic muscle.

Upon tissue injury, the cues released by the inflammatory component of the regenerative environment instruct somatic stem cells to repair the damaged area [11]. The elucidation of the molecular events underpinning the interplay between the inflammatory infiltrate and tissue progenitors is crucial to devise new strategies toward implementing regeneration of diseased or injured tissues. Regeneration of diseased muscles relies on muscle stem cells (satllite cells) located under the basal lamina of muscle fibers [12], which are activated in response to cytokines and growth factors [13]. The current lack of knowledge of how

external cues coordinate gene expression in these cells precludes their selective manipulation through pharmacological interventions.

The inflammatory infiltrate is a transient, yet essential, component of the satellite cell niche and provides the source of locally released cytokines, such as interleukin (IL)-1, IL-6, and tumor necrosis factor-α (TNF-α), which regulate muscle regeneration [14]. As an inducible element of the satellite cell niche, the inflammatory infiltrate provides an ideal target for selective interventions aimed at manipulating muscle regeneration [15]. However, because local inflammation regulates multiple events within the regeneration process, global anti-inflammatory interventions have both positive and negative effects on satellite cells [16]. Thus, it is important to elucidate the intracellular signaling by which inflammatory cytokines deliver information to individual genes in satellite cells.

Similarly to the embryonic stem cells that build organs, adult stem cells that regenerate organs are capable of symmetric and asymmetric division, self-renewal, and differentiation. This precise coordination of complex stem cell responses throughout adult life is regulated by evolutionally conserved signaling networks that cooperatively direct and control (1) the breakage of stem cell quiescence, (2) cell proliferation and self-renewal, (3) cell expansion and prevention of premature differentiation and finally, (4) the acquisition of terminal cell fate. This highly regulated process of tissue regeneration recapitulates embryogenic organogenesis with respect to the involvement of interactive signal transduction networks such as hepatocyte growth factor (HGF), Notch, MyoD, calcineurin, and SRF [17, 18]. This review aims to outline the molecular and cellular mechanisms of muscle regeneration.

2. Early immune response

Two distinct macrophage populations exist. Classically activated (or type I) macrophages are induced by interferon (IFN)-γ, alone or in concert with microbial stimuli (e.g. lipopolysaccharide) or selected cytokines (e.g. TNF-α and granulocyte macrophage colony-stimulating factor). They have pro-inflammatory functions: classically activated macrophages produce effector molecules (reactive oxygen and nitrogen intermediates) and inflammatory cytokines (IL-1β, TNF-α, IL-6), participate as inducer and effector cells in polarized Th1 responses, and mediate resistance against intracellular parasites and tumors. Type I macrophages characteristically and selectively express pro-inflammatory chemokines, in particular CCL [chemokine (C-C motif) ligand] 3. Alternatively activated (or type II) macrophages comprise cells exposed to IL-4 or IL-13, immune complexes, IL-10, and glucocorticoid; they participate in polarized Th2 reactions, promote killing and encapsulation of parasites, and are present in established tumors, where they promote progression. Moreover, alternatively activated macrophages are involved in wound healing and have immunoregulatory functions [18]. The expression of membrane receptors, like the hemoglobin scavenger receptor CD163, unambiguously identifies type II macrophages [19].

Studies in the rat have shown that type I macrophages are associated with muscle necrosis, whereas type II macrophages are associated with regenerative myofibers [20]. Of striking

interest, these cells, once within the muscle, apparently acquire a type II phenotype, revealing a previously ignored plasticity. What are the signals that trigger the shift? Recognition and phagocytosis of muscle cell debris is probably a critical event. Indeed while type I macrophages enhance the proliferation of local myogenic precursor cells, type II macrophages stimulate their fusion and differentiation [21]. Some molecular interactions are required for macrophage recruitment and function in damaged muscles. The muscle tissue of mice with a null mutation of CCR2, the CCL2 receptor, undergoes regenerating defects including fibrosis and calcification after muscle damage. In addition, uPA (urokinase-type plasminogen activator)-/- macrophages fail to infiltrate damaged muscle [22]. This failure is associated with defective muscle regeneration, demonstrating that uPA is required for the homeostatic response to injury. Mice lacking an inhibitor of uPA, PAI-1 (plasminogen activator inhibitor 1), exhibit increased uPA activity: injured muscle of PAI-1-/- mice shows evidence of increased macrophage accumulation, and of accelerated muscle repair [23]. Expression of uPA is apparently required for the expression of insulin-like growth factor-I (IGF-I), a central regulator of muscle regeneration [24]. IGF-I suppresses the expression and activity of macrophage migration inhibitory factor and the transcription factor NF-κB, possibly directly regulating the persistence of inflammatory responses [25, 26].

3. Hepatocyte growth factor and neuronal nitric oxide synthase

By 24 hours after muscle injury, satellite cells enter the G1/S phase of the cell cycle [27]. Two factors have been demonstrated to activate quiescent satellite cells. The first is HGF. Early experiments using single muscle fibers with associated quiescent satellite cells have shown that growth factors, such as IGF-I and fibroblast growth factor (FGFs), do not activate satellite cells in fibers [28, 29]. Although IGF-I and FGFs are reported to activate satellite cells, the studies involved typically used cultures of muscle cells that were not quiescent; IGF-I and FGFs increase the proliferative activity of satellite cells once they are activated, even when that activation results during the cell isolation process, i.e. prior to the plating of cells or fibers for culture. Moreover, platelet-derived growth factor BB, transforming growth factor-β (TGF-β), and epidermal growth factor do not stimulate quiescent cells to enter the cell cycle *in vitro* [30, 31]. Therefore, HGF is the only growth factor that has been established to have the ability to stimulate quiescent satellite cells to enter the cell cycle early in a culture assay and *in vivo* [32, 33]. HGF is localized to the extracellular domain of un-injured skeletal muscle fibers through a possible association with glycosaminoglycan chains of proteoglycans that are essential components of the extracellular matrix, and following injury, quickly associates with satellite cells [34] by binding to its receptor, c-Met [33].

The second component shown to be involved in satellite cell activation is nitric oxide (NO), possibly through activation of matrix metalloproteinases (MMP), which induce the release of HGF, from the extracellular matrix [34, 35]. Studies *in vitro* and *in vivo* using rodent muscle have shown HGF and NO to regulate the activity of many satellite cells [33, 34, 36, 37]. Intriguingly, inhibition of NO production inhibits HGF release, c-Met/HGF co-

localization, and satellite cell activation [34]. NO is a short-lived free radical that is well known as a freely diffusible and ubiquitous molecule produced by nitric oxide synthase (NOSs) from the L-arginine of substrates. In skeletal muscle, neuronal NOS (nNOS, also called NOS-1) is localized to the sarcolemma of muscle fibers by association at its amino terminus with alpha1-syntrophin linked to the dystrophin cytoskeleton [38]. The NO radical is normally produced in very low level pulses by muscles under conditions where satellite cells are quiescent [39], and the expression and activity of constitutive NOS (nNOS and eNOS) are up-regulated by exercise, loading injury, shear force, and mechanical stretch. NO also induces expression of follistatin [40], a fusigenic secreted molecule, known to antagonize myostatin, thus possibly contributing to the exit of satellite cells from quiescence.

More recently, Tatsumi and Allen [37] proposed the intriguing hypothesis that HGF has another role in satellite cells. Although, in culture, a low level of HGF (2.5 ng/ml) optimally stimulates the activation of satellite cells, high levels of HGF (10-500 ng/ml) promote the re-entering of quiescence through a concentration-dependent negative feedback mechanism. Such a role seems to be regulated by the induction of the cyclin-dependent kinase (CDK) inhibitor p21 in a myostatin-dependent manner. Further descriptive analysis is needed to elucidate whether HGF and myostatin really do interact in skeletal muscle *in vivo*. Tatsumi and Allen [37] suggested the importance and difficulty of monitoring whether or not extracellular HGF concentrations reach a threshold (over 10 ng/ml) in muscle of living animals.

4. The proliferating process of satellite cells

4.1. Leukemia inhibitory factor

Leukemia inhibitory factor (LIF) is a newly discovered myokine [41], originally identified by its ability to induce the terminal differentiation of myeloid leukemic cells. Today, LIF is known to have a wide array of functions, including acting as a stimulus for platelet formation, the proliferation of hematopoietic cells, bone formation, neural survival and formation, muscle satellite cell proliferation and acute phase production by hepatocytes [42]. LIF is a long chain four α-helix bundle cytokine, which is highly glycosylated and may be present with a weight of 38-67 kDa, which can be deglycosylated to ~20 kDa [43, 44]. Several tissues, including skeletal muscle, express LIF. LIF is constitutively expressed at a low level in type I muscle fibers [45, 46] and is implicated in conditions affecting skeletal muscle growth and regeneration [45-47]. Indeed, LIF knockout mice showed a decrease in the area occupied by regenerating myofibers after crush injury compared to wild-type mice, which was restored by administration of exogenous LIF [48]. Administration of LIF to the site of crush injury in wild-type mice increased the area occupied by regenerating fibers with an associated increase in average myofiber diameter [48, 49]. These original studies suggested that enhanced regeneration and increases in fiber size occurred, at least in part via stimulation of the proliferation of muscle-forming myoblast cells, thus providing more cells to fuse to and increase the size of regenerating fibers.

In 1991, Austin and co-workers demonstrated that LIF stimulated myoblast proliferation in culture [50], thereby showing that LIF functions as a mitogenic growth factor when added to muscle precursor cells *in vitro*. To date, different groups have confirmed this finding and shown that LIF induces satellite cell and myoblast proliferation, while preventing premature differentiation, by activating a signaling cascade involving Janus kinase 1 (JAK1), signal transducer and activator of transcription (STAT) 1, and STAT3 [51, 52]. In line with this, the specific LIF receptor is primarily expressed by satellite cells and not by mature muscle fibers [53]. Thus, it seems that LIF has the potential to affect satellite cells rather than mature muscle fibers.

Earliest descriptions of LIF as a possible mitogen for myoblasts suggested that LIF treatment increased the number of human and mouse-derived primary myoblast cells in a dose-dependent manner after several days of culture, with the earliest increases noticeable after 6 days [50, 54]. There is evidence to suggest that LIF promotes survival of myoblasts and other cell types [55, 56]. Hunt et al. [57] found that LIF treatment significantly reduced staurosporine-induced apoptotic DNA fragmentation by 37% and also reduced the proteolytic activation of caspase-3 by 40% compared to controls. This apoptosis-inhibiting role of LIF was completely abolished by a PI3-K (phosphatidylinositol 3-kinase) inhibitor (wortmannin). Therefore, LIF appears to increase the number of satellite cells by promoting proliferation and blocking apoptosis.

4.2. Insulin-like growth factor-I and MAPK (proliferation phase)

The anabolic effects of IGF-I have been demonstrated in both muscle cell lines and animal models [58-60]. For example, the addition of IGF-I to cultured myotubes results in an enlargement of myotube diameters and a higher protein content, while the delivery of IGF-I either through osmotic pumps or genetic overexpression results in increased muscular mass in rodents [24, 58]. Mechanical loading also results in skeletal muscle synthesis of IGF-I [61, 62] *in vivo*, which stimulates gene expression, DNA and protein synthesis, different transport mechanisms, migration, proliferation, and differentiation [63]. Therefore, investigators conclude that IGF-I is a critical factor involved in skeletal muscle hypertrophy in vivo as well as in cultured myotube enlargement in vitro.

IGF-I is thought to induce muscle growth through the increased proliferation of satellite cells and the enhancement of protein translation resulting in an increase in the rate of protein synthesis [63, 64]. In addition to stimulating myoblast proliferation, IGF-I stimulates myoblast differentiation [65]. For example, IGF-I inhibits production of myogenin, a protein that stimulates muscle cell differentiation, thus allowing increased myoblast proliferation. It is known that the binding of IGF-I to its receptor, after tyrosine (auto)phosphorylation of the receptor, results in the initiation of intracellular cascades of various kinase systems. However, the interplay between the elements of these intracellular signaling pathways has been described based on results of experiments with skeletal muscle cell types of different species and under various conditions. Namely, in mouse and rat skeletal muscle preparations, the involvement of both the MAPK (mitogen-activated protein kinase)

pathway and MAPK-independent signaling mechanisms, including PI3-K/Akt and protein kinase C (PKC), was equally documented [66-68]. In primary cultured human skeletal muscle cells, Czifra et al. [69] demonstrated that the proliferation-enhancing effect of IGF-I was completely inhibited by the PKCδ-specific inhibitor Rottlerin but not by inhibitors of the "conventional" PKCα and γ isoforms or by inhibitors of the MAPK or PI3-K pathway. In addition, overexpression of a kinase inactive mutant of PKCδ prevented the proliferating action of IGF-I. Furthermore, they showed, in mouse C2C12 cells, that the MAPK inhibitor PD098059 partially inhibited the action of IGF-I. Taken together, these results demonstrate a novel, central and exclusive involvement of PKCδ in mediating the action of IGF-I in human skeletal muscle cells, with an additional yet PKCδ-dependent contribution of the MAPK pathway in C2C12 myoblasts.

4.3. Notch-dependent signaling

The proliferating process in satellite cells appears to be controlled by Notch signaling during muscle regeneration [70]. Within hours to days following muscle injury, there is increased expression of Notch signaling components (Delta-1, Notch-1 and active Notch) in activated satellite cells and neighboring muscle fibers [8, 70]. Up-regulation of Notch signaling promotes the transition from activated satellite cells to highly proliferative myogenic precursor cells and myoblasts, as well as prevents differentiation to form myotubes [8, 71, 72]. Proliferation was decreased and differentiation was promoted when Notch activity was inhibited in myoblasts with a Notch antagonist, Numb, a gamma-secretase inhibitor, or with small-interfering RNA (siRNA) knockdown of presenilin-1 [70, 71, 73]. In addition, mutations in Delta-like 1 or CSL result in excessive premature muscle differentiation and defective muscle growth [74]. Apparent impairment of Notch signaling occurs in aged muscle, because expression of the Notch ligand, Delta, is not upregulated following injury in this muscle. Forced activation of this pathway with a Notch-activating antibody can restore the regenerative potential by inducing the expression of several positive regulators (PCNA, Cyclin D1) of cell cycle progression [8, 9].

A recent study revealed that levels of TGF-β are higher in aged than young satellite cell niches [10]. Further analysis showed greater activation of the TGF-β pathway in old satellite cells, and physical competition between Notch and pSmad3 at the promoters of multiple CDK inhibitors [10, 75]. Furthermore, the decline of Notch1 signaling with age is thought to be another cause of the decreased regenerative potential of aged skeletal muscle. Indeed, enhancement of Notch-1 signaling promotes muscle regeneration in old skeletal muscle [8, 9]. Although these experiments suggest a crucial role for Notch1 signaling in satellite cell function, much remains to be determined, especially regarding the role of Notch3 signaling during muscle regeneration. Notch3 was expressed in satellite cells, and various structural and functional differences between Notch3 and Notch1/Notch2 have been reported [76]. More recently, Kitamoto and Hanaoka [77] conducted two very intriguing experiments. They analyzed muscle after repeated injuries, by generating mice deficit in Notch3 and also by repetitive intramuscular injections of cardiotoxin (CTX) into the Notch3-deficient mice.

They found a remarkable overgrowth of muscle mass in the Notch3-deficient mice but only when they suffered repetitive muscle injuries. Analysis of cultured myofibers revealed that the number of self-renewing Pax7-positive satellite cells attached to myofibers was increased in the Notch3-deficient mice compared to control mice. Given these findings, the Notch3 pathway might act as a Notch1 repressor by activating Nrarp, a negative feedback regulator of Notch signaling.

5. The differentiation of satellite cells

5.1. MyoD family

Satellite cell myogenic potential mostly relies on the expression of Pax genes and myogenic regulatory factors (MRFs: MyoD, Myf5, myogenin, and MRF4). Sequential activation and expression of Pax3/7 and MRFs is required for the progression of skeletal myoblasts through myogenesis. Pax7 is expressed by all satellite cells and essential to their postnatal maintenance and self-renewal [78]. Pax7 induces myoblast proliferation and delays their differentiation not by blocking myogenin expression [79] but by regulating MyoD [80]. In parallel, myogenin directly down-regulates Pax7 protein expression during differentiation [80]. MyoD is required for the differentiation of skeletal myoblasts [81, 82]. In addition, MyoD null satellite cells showed reduced myogenin expression and absolutely no MRF4 expresion, and displayed a dramatic differentiation deficit [82]. Indeed, muscle regeneration *in vivo* is markedly impaired in MyoD null mice [83]. In contrast, Myf5 regulates the proliferation rate and homeostasis [84]. MyoD can compensate for Myf5 in adults. Myf5 deficiency leading to a lack of myoblast amplification and loss of MyoD induced an increased propensity for self-renewal rather than progression through myogenic differentiation. The differentiation factors myogenin and MRF4 are not involved in satellite cell development or maintenance [84] but induction of myogenin is necessary and sufficient for the formation of myotubes and fibers.

5.2. IGF-I and calcineurin-dependent signaling

IGF-I positively regulated not only the proliferation but also the differentiation of satellite cells/myoblasts *in vitro* possibly through a calcineurin-dependent pathway. Since activated calcineurin promotes the transcription and activation of myocyte enhance factor 2 (MEF2), myogenin, and MyoD [85-87], calcineurin seems to control satellite cell differentiation and myofiber growth and maturation, all of which are involved in muscle regeneration [88, 89]. In fact, our previous study [88] showed a marked increase in the amount of calcineurin protein and the clear colocalization of calcineurin and MyoD or myogenin in many myoblasts and myotubes during muscle regeneration. In addition, we showed that the inhibition of calcineurin by cyclosporine A (CsA) induced extensive inflammation, marked fiber atrophy, and the appearance of immature myotubes in regenerating muscle compared with placebo-treated mice [88]. Several other studies indicated such defects in skeletal muscle regeneration when calcineurin was inhibited [90, 91], whereas transgenic activation

of calcineurin is known to markedly promote the remodeling of muscle fibers after damage [92, 93].

Many researchers have utilized CsA, though in different amounts, to determine the downstream modulators of calcineurin signaling. We found that intraperitoneal CsA treatment daily at 25 mg/Kg/day enhanced the expression of myostatin and Smad3 mRNA in regeneration-defective tibialis anterior muscle after an injection of bupivacaine [89]. The possibility that myostatin is a downstream mediator of calcineurin signaling has been indicated by experiments with two different transgenic mice [94]. In addition, calcineurin's pharmacological inhibition caused a decline in the transcription and activation of myogenin and MyoD during myogenic differentiation by a downregulation of MyoD expression [95]. Considering these findings, calcineurin seems to block the myostatin-Smad3 pathway to enhance the expression of myogenic differentiation factor (MyoD) during muscle regeneration *in vivo*. Using CsA treatment *in vivo*, recent evidence including that obtained by our group has also identified Id1 [87, 89], Id3 [87], and Egr-1 [87] as a possible downstream negative hypertrophic effector target of the calcineurin-NFAT (nuclear factor of activated T-cells) pathway.

FOXO (forkhead box O)-induced expression of Atrogin-1 has been shown to inhibit calcineurin activity [96]. More recently, the calcineurin variant CnAβ1 was suggested to block the nuclear localization of the FOXO protein and the expression of several genes targeted by FOXO [the muscle ring finger-1 (MuRF1), Gadd45a, Pmaip1, and atrogin genes] in C2C12 myoblasts [93]. In addition, transgenic up-regulation of CnAβ1 expression promotes the remodeling of cardiotoxin-treated muscle fibers [93]. In cardiomyocytes, calcineurin directly binds and dephosphorylates (inactivates) Akt; FOXO indirectly activates Akt by inhibiting calcineurin phosphatase activity [97]. In murine C2C12 myotubes, Akt was shown to antagonize calcineurin signaling by causing hyperphosphorylation of NFATc1 [60]. Interaction between CnAβ1 and FOXO during muscle regeneration is a very attractive idea, although it has not been demonstrated in adult skeletal muscle *in vivo*.

5.3. Serum response factor

SRF is an ubiquitously expressed member of the MADS box transcription factor family, sharing a highly conserved DNA-binding/dimerization domain, which binds the core sequence of SRE/CArG boxes [CC (A/T)6 GG] as homodimers [98]. Functional CArG boxes have been found in the cis-regulatory regions of various muscle-specific genes, such as the skeletal α-actin [99], muscle creatine kinase, dystrophin, tropomyosin, and myosin light chain 1/3 genes. The majority of SRF's targets are genes involved in cell growth, migration, cytoskeletal organization, and myogenesis [100, 101]. SRF was first shown to be essential for both skeletal muscle cell growth and differentiation in experiments performed with C2C12 myogenic cells. In this model, SRF inactivation abolished MyoD and myogenin expression, preventing cell fusion in differentiated myotubes [102]. SRF also enhances the hypertrophic process in muscle fibers after mechanical overloading [103]. For example, we showed that, in mechanically overloaded muscles of rats, SRF protein is co-localized with MyoD and

myogenin in myoblast-like cells during the active differentiation phase [104]. Recent results obtained with specific SRF knock-out models, by the Cre-LoxP system, emphasize a crucial role for SRF in postnatal skeletal muscle growth and regeneration [105], by direct binding of IL-4 and IGF-I promoters *in vivo*. These lines of evidence appear to indicate that SRF modulates the differentiating process of satellite cells in adult mature muscle.

The expression and cellular localization of SRF and myocardin-related transcription factor-A (MRTF-A) appear to be regulated by several upstream factors including β1-integrin, RhoA, striated muscle activators of Rho signaling (STARS) [106], and MuRF2 [107]. For instance, Lange et al. [107] demonstrated that SRF is blocked and relocalized by the nuclear translocation of MuRF2, which regulates a signaling pathway composed of titin-Nbr1-p62/SQSTM1 at the position of the sarcomere depending on mechanical activity. To date, there has been no attempt to investigate whether titin-Nbr1-p62/SQSTM1 and MuRF2 affect muscle regeneration. In addition, the mutation of SRF delineated the translocational action of MRTF-A induced *in vitro* by STARS, a muscle-specific actin-binding protein [106].

5.4. Wnt-dependent signaling

Similar to Notch signaling, canonical Wnt signaling is critical for muscle repair [108-111]. The canonical Wnt signaling cascade requires soluble Wnt ligands to interact with Frizzled receptors and low-density lipoprotein receptor-related protein co-receptors (LRP). This coordination stimulates phosphorylation of Disheveled and inactivates GSK3β's phosphorylation of β-catenin. In the nucleus, the de-phosphorylated β-catenin binds to T-cell factor/Lymphoid enhancer factor-1 transcription factors [112], which may directly activate Myf5 and MyoD or may upregualte MRF co-activators such as c-Jun N-terminal kinases [113, 114]. It is suggested that Notch activity presides during myoblast proliferation after which there is a temporal switch to Wnt signaling and subsequent myoblast differentiation and fusion into myotubes [108]. Inhibiting Notch (with soluble Jagged ligand or with a γ-secretase inhibitor) or activating Wnt (by inhibiting GSK3β or adding Wnt3a) decreases Myf5 expression and promotes muscle differentiation providing evidence that Notch signaling needs to be turned off and Wnt turned on for differentiation to ensue [108, 115].

This hypothesis was supported by the finding that aberrant activation of the Wnt pathway can lead to fibrogenic conversion of cells in different lineages [116-118]. In fact, Wnt signaling was shown to be enhanced in aged muscle and in myogenic progenitors exposed to aged serum [116]. To directly test the effects of Wnt on cell fate and muscle regeneration, Brack et al. [116] altered Wnt signaling *in vitro* and *in vivo.* Addition of Wnt3A protein to young serum resulted in increased myogenic-to-fibrogenic conversion of progenitors *in vitro* [116]. Conversely, the myogenic-to-fibrogenic conversion of aged serum was abrogated by Wnt inhibitors [116]. *In vivo*, the injection of Wnt3A into young regenerating muscle 1 day after injury resulted in increased connective tissue deposition and a reduction in satellite cell proliferation [116]. The authors therefore tested whether inhibiting Wnt signaling in aged muscle would reduce fibrosis and enhance muscle regeneration.

6. Second-stage fusion (late differentiation)

Growth and maturation of the muscle cells are achieved through a second-stage fusion, which occurs between the nascent myofibers/myotubes and myoblasts. Although many regulators of this fusion process have been revealed in recent years [119], a better understanding of the regulation is still needed. mTOR (mammalian target of rapamycin) is one of the candidates regulating the fusion. mTOR signaling regulates a wide range of biological processes, including cell growth, various types of cellular differentiation, and metabolism [120, 121]. mTOR assembles two biochemically and functionally distinct protein complexes, mTORC1 (mTOR complex 1) and mTORC2, which are sensitive and insensitive to rapamycin, respectively [120]. Rapamycin-sensitive mTORC1 signaling has emerged as a key regulator of skeletal muscle differentiation and remodeling. Rapamycin inhibits myoblast differentiation *in vitro* [122, 123], compensatory myofiber hypertrophy *in vivo*, and regrowth of myofibers after atrophy [124]. The regulation of skeletal myocyte differentiation by mTORC1 occurs at two stages via distinct mechanisms. mTORC1 controls the initiation of myoblast differentiation by regulating IGF-II expression [123], whereas late-stage myocyte fusion leading to myotube maturation is regulated by mTORC1 through a yet to be identified secreted factor [125]. More recent findings pointed out that the fusion factor targeting mTORC1 is follistatin during the late differentiation phase. Sun et al. [126] have found that, in C2C12 cells differentiating for 24-72h, miR-1 luciferase (enhancer) activity was markedly downregulated after treatment with rapamycin but not wortmannin (PI3-K inhibitor) or SB203580 (MAPK inhibitor). In addition, rapamycin increased the amount of histone deacetylase 4 (HDAC4) protein and reduced follistatin mRNA and MyoD protein levels in C2C12 and C3H10T1/2 cells. Furthermore, daily administration of trichostatin A and a single dose of adenovirus expressing follistatin rescued the defective muscle regeneration caused by treatment with rapamycin. Sun et al. [126] proposed the intriguing hypothesis that mTOR-miR-1 promotes myocyte fusion by recruiting HDAC4-follistatin during myoblast differentiation *in vitro* and skeletal muscle regeneration *in vivo.*

7. Satellite cell self-renewal

A hallmark of stem cells is their ability to self-renew. In skeletal muscle, asymmetric cell division takes place in a subset of the satellite cell population to generate a self-renewing progenitor and hyperplastic daughter cell which later contributes to *de novo* muscle formation [127]. Several extrinsic pathways have been implicated in mediating this phenomenon [108, 127, 128]. One family of candidate peptides is the Wnt family of signaling molecules which consists of over 19 cysteine-rich secreted glycoproteins that in part bind the Frizzled (Fzd) receptors [129].

In a non-canonical Wnt cascade, Wnt7a has been characterized for its role as the extracellular ligand mediating asymmetric cell division which is thought to be the mechanism by which satellite cells are able to self-renew [128]. Lineage tracing of satellite cell populations indicates ~90% of cells to have at some point expressed Myf5 (Pax7+Myf5+) [127]. The Myf5+ cells have a reduced potential to self-renew as the majority undergo

symmetrical cell divisions and later contribute to muscle syncitia [127]. The remaining ~10% of satellite cells divide asymmetrically and give rise to Pax7+Myf5- as well as Pax7+Myf5+ progeny thereby maintaining the stem cell pool of muscle progenitors [127]. The capacity of Pax7+Myf5- cells to self-renew is explained by expression of the Wnt receptor Fzd7 on these cells but not on Pax7+Myf5+ cells, thus allowing induction of asymmetrical cell division via Wnt7a-induced signaling [128]. Importantly, stimulation of satellite cells with Wnt7a leads to an increase in the symmetrical expansion of satellite cells, while muscle from Wnt7a knockout mice displays a dramatic reduction in satellite cell numbers following regeneration [128].

The Notch inhibitor, Numb is also asymmetrically expressed on the activated satellite cells and may regulate cell fate choices by promoting progression down the myogenic lineage [130]. Self renewal may also occur through symmetrical division in which both daughter cells maintain stem-cell properties [131, 132]. Cells that do not express MyoD but continue to express Pax7 are suggested to be refrained from self-renewal [133].

8. Other regulators of the muscle regenerating process

8.1. Myostatin and TGF-β

The TGF-β superfamily plays a crucial role in normal physiology and pathogenesis in a number of tissues. Myostatin was first discovered during screening for novel members of the TGF-β superfamily, and shown to be a potent negative regulator of muscle growth [134]. Like other TGF-β family members, myostatin is synthesized as a precursor protein that is cleaved by furin proteases to generate the active C-terminal dimer. When produced in Chinese hamster ovary cells, the C-terminal dimer remains bound to the N-terminal propeptide, which remains in a latent, inactive state [135]. Most, if not all, of the myostatin protein that circulates in blood also appears to exist in an inactive complex with a variety of proteins, including the propeptide [136]. Myostatin binds to and signals through a combination of Activin IIA/B receptors on the cell membrane, but has higher affinity for ActRIIB. On binding to ActRIIB, myostatin forms a complex with a second surface type I receptor, either activin receptor-like kinase (ALK4 or ActRIB) or ALK5 to stimulate the phosphorylation of receptor Smad and the Smad2/3 transcription factors in the cytoplasm. This leads to the assembly of Smad2/3 with Smad4 to form a heterodimer that is able to translocate to the nucleus and activate the transcription of target genes [137].

Studies indicate that myostatin inhibits the activation, differentiation, and self-renewal of satellite cells [138-140] and the expression of the muscle regulatory factors crucial for the regeneration and differentiation of myofibers [138, 141]. One of the known downstream targets of Smad signaling is MyoD. Interestingly, myostatin downregulates MyoD expression in an NF-κB-independent manner [142]. Myostatin also inhibits Pax3 expression, which is possibly an upstream target of MyoD [142]. Recently, it was found that FOXO1 and Smad synergistically increase the expression of myostatin mRNA and its promoter activity

in C2C12 myotubes [95]. Taken together, myostatin-mediated signaling activates FOXO, and this leads to the expression of ubiquitin ligases.

TGF-β1 is expressed during myogenesis, and its spatial and temporal expression in the developing connective tissue is correlated with the fiber-type composition of the surrounding myotubes. Myotubes formed before the expression of TGF-β1 develop into slow fibers, whereas fast fibers form when myoblasts are adjacent to connective tissue expressing TGF-β1 [143]. TGF-β1 has been shown to inhibit the differentiation of fetal myoblasts but does not affect embryonic myoblasts [144]. In mature adult muscle, TGF-β negatively affects skeletal muscle regeneration by inhibiting satellite cell proliferation, myoblast fusion, and expression of some muscle specific-genes [145]. Furthermore, TGF-β1 induced the transformantion of myogenic cells into fibrotic cells after injury [146].

TGF-β1, a potent regulator of tissue wound healing and fibrosis, is physiologically upregulated in regenerating skeletal muscle after injury and exercise and is thought to participate in a transient inflammatory response to muscle damage [147, 148]. Persistent exposure to the inflammatory response leads to an altered extracellular matrix and increased levels of growth factors and cytokines, including TGF-β1, which contribute to the formation of fibrotic tissue [147, 148]. Increased levels of TGF-β1 inhibit satellite cell activation and impair myocyte differentiation [145, 149]. Figure 1 summarizes the calcineurin-, myostatin-, and Akt-dependent signaling in muscle regeneration.

8.2. TNF-α signaling

TNF-α has long been viewed as the quintessential proinflammatory cytokine, capable of classical activation of macrophages to the M1 phenotype, and thereby inducing the production of other proinflammatory, Th1 cytokines. Following muscle injury, the early invading neutrophil and macrophage populations express TNF-α [152], suggesting that the cytokine may contribute to the early inflammatory stages that precede muscle regeneration. TNF-α levels in muscle following acute injury peak at 24h postinjury, which indicates that TNF-α production is most tightly coupled with the Th1 inflammatory response in injured muscle [153]. Because findings show that TNF-α induces iNOS expression in myeloid cells and that myeloid cell-derived NO can cause muscle fiber damage early on, Th1 inflammatory cells have been associated with muscle damage. However, TNF-α levels remain elevated for nearly 2 weeks following acute injury, indicating that TNF-α may also modulate the regenerative process [153]. Intriguingly, the expression of TNF-α receptors by muscle cells themselves is elevated as a later consequence of injury, during the regenerative process, and enables TNF-α to act directly on muscle cells to modulate their proliferation and differentiation [152].

Numerous experimental observations indicate that TNF-α acts directly on muscle cells in affecting muscle regeneration. For example, TNF-α null mutants and TNF-α receptor mutants show lower levels of MyoD and MEF2 expression than wild-type controls following acute injury [153, 154]. The application of exogenous TNF-α to myoblasts *in vitro*

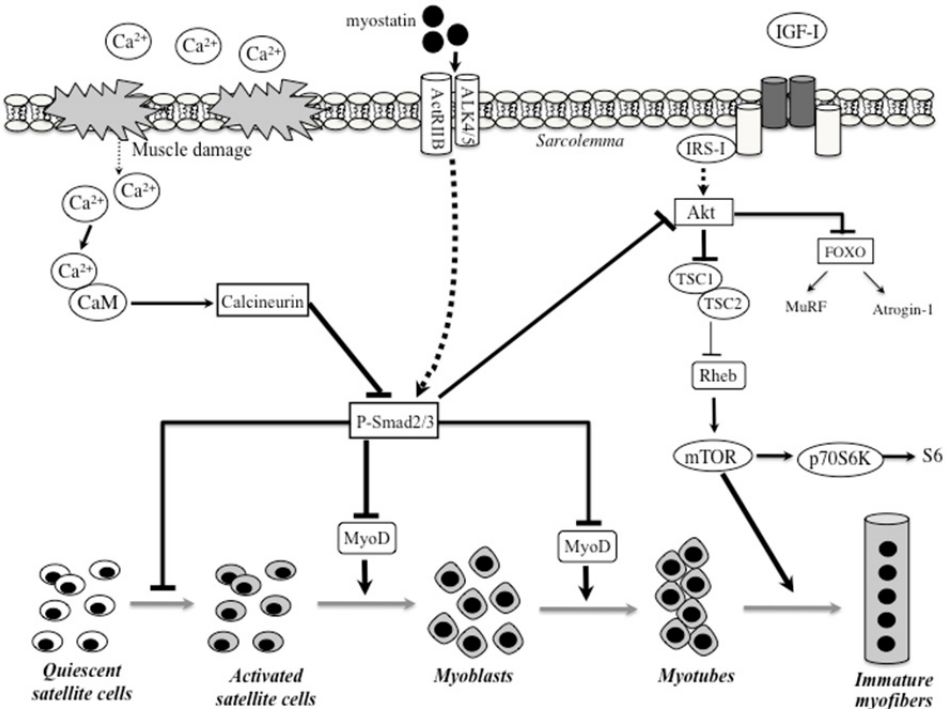

Figure 1. Schematic diagram of calcineurin-, myostatin-, and Akt-dependent signaling in muscle regeneration. Myostatin acts through activin receptor IIB (ActRIIB). The ALK4/5 heterodimer activates Smad2/3 with blocking of MyoD transactivation in an autoregulatory feedback loop. In addition, Smad3 sequesters MyoD in the cytoplasm to prevent it from entering the nucleus and activating the stem cell population. In proliferating myoblasts, this pathway arrests cell proliferation and differentiation. Moreover, recent findings [150, 151] suggest that the myostatin-Smad pathway inhibits protein synthesis probably by blocking the functional role of Akt. Damage the muscle fiber membranes after treatment with myotoxin elicits an increase in intracellular Ca^{2+} levels via the influx of Ca^{2+} from the extracellular space. Binding of the Ca^{2+}/CaM complex to the calcinuerin regulatory subunit leads to its activation. Activated calcineurin dephosphorylates a range of transcription factors (including MEF2 and NFAT). Activated calcineurin inhibit the functional role of Egr-1 and Smad2/3 [87, 89], and promotes myogenic differentiation. Calcineurin signaling is markedly inhibited by myostatain [94] and FOXO [96, 97]. IGF-I produced by the regenerating muscle activates PI3-K-Akt-mTOR signaling resulting in a positive protein balance. One part of mTOR (mTORC1) enhances myotube differentiation at later stages probably through the induction of follistatin expression. CaM; calmodulin, IRS-1; insulin receptor substrate-1

increases their proliferation, and inhibited the process of early differentiation to terminal differentiation [155-157]. Experiments *in vivo* using a lung-specific TNF-α transgene also showed a differentiation-inhibiting role [158]. These TNF-α abundant mice exhibited attenuated expression of developmental myosin heavy chain (MHC) in reloaded soleus muscle after hindlimb suspension [158]. TNF-α affects several intracellular signaling pathways leading to the activation of NF-κB, caspase 8, and stress-induced factors like c-Jun N-terminal kinase (JNK) and p38 MAPK [159]. Activation of NF-κB can inhibit myogenesis

through several processes. NF-κB can promote the expression and stability of cyclin D1 in muscle [155], leading to increased cell proliferation and inhibition of differentiation. Furthermore, NF-κB can cause destabilization of MyoD mRNA and degradation of MyoD protein [155, 156]. The role of JNK in the effect of TNF-α on myogenesis has been less investigated. A recent study suggested that activation of JNK by TNF-α blocks IGF-I signaling necessary for the differentiation of myoblasts [160].

TNF-α can activate signaling through other pathways independent of NF-κB to promote muscle differentiation. Both IL-1 and TNF-α can activate p38 kinase [161], promoting the differentiation. In particular, inhibition of p38 in skeletal muscle cells *in vitro* inhibits myocytes from fusing to form myotubes and reduces the expression of MEF2, myogenin, and myosin light chain kinase [162], all of which indicate that p38 activation can promote muscle differentiation. Furthermore, p38 activation can also increase the activity of MyoD [162, 163]. The ability of p38 to promote myogenesis relies, in part, on its ability to phosphorylate and increase the transcriptional activity of MEF2 [162, 164]. In contrast, p38 activation can also inhibit myogenesis by the phosphorylation of other MyoD family members (MRF4). The elevated expression and activity of p38 late in muscle differentiation leads to increased MRF4 phosphorylation and, as a consequence, a decline in desmin and skeletal α-actin expression [165]. In fact, overexpression of MRF4 in a transgenic mouse line caused defective muscle regeneration following injury [166]. Therefore, TNF-α-dependent signaling regulates various aspects of the muscle regenerating process (immune response, and proliferation and differentiation of satellite cells) through different downstream mediators (NF-κB, JNK, and p38) [Figure 2].

8.3. TWEAK

TNF-like weak inducer of apoptosis (TWEAK) is a pro-inflammatory cytokine belonging to the TNF superfamily of ligands. Initially synthesized as a type II transmembrane protein, TWEAK is cleaved to its soluble form, and signals as a trimerized molecule [167]. Generally, TWEAK signaling occurs through binding to Fn14, a type I transmembrane receptor belonging to the TNF receptor superfamily. TWEAK has been found to promote the regeneration and growth of myofibers after injury [168-170]. Dogra et al. [168] reported that TWEAK inhibits the differentiation of cultured C2C12 or primary myoblasts into multinucleated myotubes. More recently, a transgenic model of TWEAK also suggested a differentiation-promoting role in muscle regeneration *in vivo*. In fact, mRNA levels of TNF-α, IL-6 and CCL-2 and protein levels of embryonic MHC were significantly reduced in cardiotoxin (CTX)-injected TA muscle of TWEAK-KO mice compared to that of wild-type mice [171]. In addition, these parameters were found to be significantly inceased in regenerating TA muscle of TWEAK-Tg mice compared to that of control mice. Since such a modulation of the TWEAK gene caused no apparent differences in levels of phospho-Akt and phospho p38MAPK in the regenerating muscle among each mouse model, TWEAK seems to function independently of Akt- and p38-linked signaling [171]. Intriguingly, electromobility shift asay by Mittal et al. [171] indicated the possibility of TWEAK-NF-κB signaling, although further descriptive analysis needs to be done.

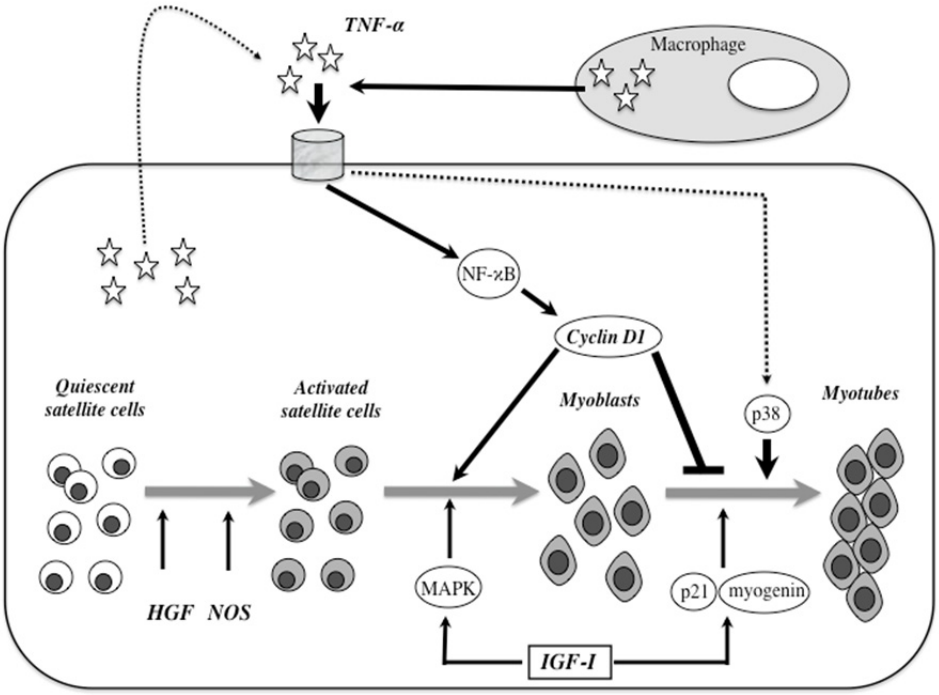

Figure 2. The functional role of TNF-α signaling in the regenerating muscle. HGF and nNOS co-ordinate the switch from quiescence to activation in satellite cells. IGF-I enhances the proliferation of satellite cells via a MAPK-dependent pathway. IGF-I also promotes myogenic differentiation via p21 and myogenin. In regenerating muscle after treatment with myotoxin, the differentiating myotubes seem to be fused together and/or incorporated into the existing muscle fibers. TNF-α, which is produced by the damaged muscle and macrophages, stimulates TNFR. TNFR activates NF-κB-signaling, in turn cyclin D1 activate the proliferation, but not differentiation, of satellite cells. In addition, TNF-α activates p38-dependent signaling leading to the differentiation of myoblasts.

8.4. MicroRNAs

The human genome contains thousands of non-coding RNAs, the best-studied class of which are microRNAs (miRNAs)[172], which regulate gene expression at the transcriptional and post-transcriptional levels. miRNAs suppress gene expression through their complementarity to the sequence of one or more RNAs, usually at a site in the 3′ untranslated region. The formation of a miRNA-target complex results either in inhibition of protein translation or in degradation of the mRNA transcript through a process similar to RNA interference [173]. There is no doubt that the formation, maintenance, and physiological and pathophysiological responses of skeletal muscles, with all their complex regulatory circuits, are subject to regulation by non-coding RNAs.

Many miRNAs are expressed in skeletal and cardiac muscle. Some of them are found specifically, or at least are highly concentrated, in skeletal and/or cardiac muscle, suggesting

specific roles in myogenesis [174]. The expression of the muscle-specific miRNAs miR-1, miR-133, miR-206, and miR-208 seems to be under the control of a core muscle transcriptional network, which involves the pleiotropic SRF, MyoD, and the bHLH transcription factor Twist in cooperation with MEF2 [175-177]. Chromatin immunoprecipitation followed by a microarray analysis indicated that MyoD and myogenin bind sequences upstream of miR-1 and miR-133 [176]. miR-133a increases myoblast proliferation, via its repression of SRF [178], while miR-1 stimulates myoblast differentiation via its inhibition of histone deacetylase 4 (HDAC4) [178]. In addition, MyoD has been demonstrated to utilize miRNAs, including miR-1 and miR-206, to suppress downstream gene expression [178, 179]. More recently, Hirai et al. [180] have demonstrated that miR-1 and miR-206 bind to two miR-1/miR-206-binding sequences within the Pax3-3'UTR and suppress Pax3 expression. Since Pax3 expression increases cell survival and suppresses myogenic differentiation in myoblasts, down-regulation of Pax3 has been shown to elicit proper myogenic differentiation along with an increase in apoptosis [180]. An analogus role was described for the regulation of Pax7, which is repressed by miR-1 and miR-206 [178, 181]. In contrast, miR-221 and miR-222 are downregulated during the transition from proliferation to differentiation [182]. Decreases in these miRNAs are associated with increased expression of the cell cycle inhibitor p27. Overexpression of miR-221 and miR-222 in differentiating myotubes delays cell cycle withdrawal and differentiation, a response associated with a reduction in sarcomeric protein [182].

9. Conclusions and perspectives

In normal, skeletal muscle possesses a robust capacity to repair itself, the ability to augment and enhance this process would significantly advance the treatment of congenital muscle disorders and severe muscle trauma for which, even with the best of present-day treatments, physical handicap or amputation are the most likely outcomes. Sarcopenia seems to include the defect of muscle regeneration probably due to the repetitive muscular damage [8-10]. Currently available data show that resistance training combined with amino acid-containing supplements would be the best way to prevent age-related muscle wasting and weakness. Therefore, for these endogenous repair therapies to advance, it is essential that an understanding exists of the biochemical, cellular and mechanical cues that promote skeletal muscle repair.

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Chapter 2

The Myogenic Regulatory Factors: Critical Determinants of Muscle Identity in Development, Growth and Regeneration

Dylan Sweetman

Additional information is available at the end of the chapter

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1. Introduction

The discovery of MyoD [1] was a landmark in our understanding of the processes leading to muscle cell differentiation. In this study a single cDNA was isolated which could induce conversion of fibroblasts into muscle cells [2]. This striking finding remains one of the clearest examples of a master regulator of cell fate and has made myogenesis an excellent paradigm for the understanding of how cell fate is induced and executed.

Other related genes were soon identified and three other closely related proteins have been isolated: Myf5 [3], Myogenin [4-6] and MRF-4 [7-9], which share the ability of MyoD to activate muscle gene expression. Together these are known as the Myogenic Regulatory Factors or MRFs. All of these genes are expressed during embryonic myogenesis exclusively in myogenic cells [10-14] although there are differences in the timing and stages of myogenesis, reflecting underlying differences in the roles of the MRFs in muscle cell commitment and differentiation [15, 16].

1.1. Muscle development

In vertebrate embryos muscle is derived from paraxial mesoderm which lies adjacent to the midline of the developing embryo [17]. In the head unsegmented mesoderm produces the branchial and extra-ocular muscles [18] while some of the neck muscles are derived from more lateral occipital mesoderm [19].

Grafting experiments in avian embryos, where quail mesoderm is grafted into chick embryo hosts, have demonstrated that all the muscles of the trunk are derived from somites, segmentally repeated epithelial structures that arise from the paraxial mesoderm [20, 21]. As

they differentiate somites produce the dermomyotome, a 'C' shaped epithelium containing proliferative muscle precusors (myoblasts) that express the transcription factor Pax3 [22].

Somites can be divided into two major domains: epaxial, located dorso-medially, and hypaxial, located ventrolaterally. Muscles arising from these domains correspond to the adult epaxial and hypaxial muscles which are innervated by the dorsal and ventral ramus of the spinal cord respectively. Cells from the dermomytome migrate around the edges of the dermomyotome to form an underlying layer, the primary myotome [23, 24], where the MRFs are first expressed and muscles begin to differentiate.

The muscles of the limb are also derived from somites but are generated when myoblasts delaminate from the hypaxial dermomyotome and migrate into the forming limb bud [17, 25]. This process is regulated by production of HGF/SF from the lateral mesoderm at limb levels which induces migration of myoblasts, to maintain them in a proliferative state and to delay MRF expression [26-28]. The expression of MyoD in these different muscle groups during embryo development is shown in figure 1.

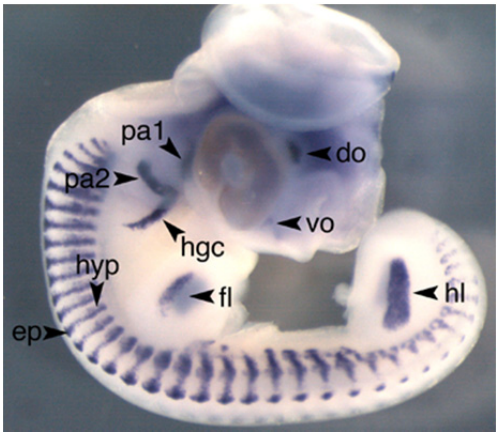

Figure 1. In situ hybridisation to show expression of MyoD in an HH stage 24 chicken embryo (approximately equivalent to mouse E11 or human Carnegie stage 16, around 40 days). Staining is seen in trunk muscle precursors in epaxial and hypaxial somites (ep, hyp), limb muscles of fore- and hindlimb buds (fl, hl), jaw and facial muscles in the pharyngeal arches (pa1, pa2), tongue muscle precursors in the hypoglossal chord (hgc) and the dorsal and ventral oblique extraocular muscles (do, vo)

Myogenesis in each of these different muscle groups, head, epaxial, hypaxial and limb, is regulated differently in the embryo [17]; however the MRFs play a key role in all of them and are part of a core transcriptional programme that operates in all skeletal muscles.

2. Regulation of the MRFs

Several signalling systems have been shown to affect MRF expression during development. It is notable that different sets of muscle precursors are regulated by separate sets of signals and, even with a single somite, there are distinct inductive pathways in hypaxial and epaxial

regions. In this section I will briefly review some of the molecular signals that have been shown to regulate MRF expression.

2.1. Signalling molecules regulating MRFs

2.1.1. Wnt

The signals induced by Wnts, the vertebrate homologues of the *Drosophila wingless* gene, are broadly divided into canonical and non-canonical types. Canonical signalling acts via β-catenin and the activation of TCF/LEF transcription factors [29] while non-canonical signalling acts via planar cell polarity or calcium dependant mechanisms [30] although these pathways are not always as clearly distinct as this division implies [31].

Explant culture of somites from chicken embryos demonstrated that signals from the neural tube and notochord are required for induction of MyoD [32] and Myf5 [33], an activity that can be recapitulated by the addition of purified Wnt-1 or Wnt-3 and low levels of Shh [34]. Mouse mesoderm explants exposed to Wnt-1 activate an epaxial, Myf5 dependant programme while exposure to Wnt-7a seems to induce a hypaxial, MyoD dependant myogenesis [35]. Wnt7 has also been implicated in the regulation of satellite cell activation via induction of MRF expression [36, 37].

In vivo Wnt1 and Wnt3a are secreted by the dorsal neural tube and are able to induce MyoD expression in the epaxial myotome, probably via β-catenin signalling [38]. In contrast, in limb muscles, Wnt-6, expressed in the limb ectoderm, has been shown to positively regulate Myf5 while downregulating MyoD [39]. In the limb induction of MRF expression is independent of β-catenin signalling although it is required for later myogenic differentiation [40].

2.1.2. Shh

The Sonic Hedgehog signalling pathway, which regulates the activity of the Gli family of transcription factors, is found in numerous inductive and patterning systems during development and plays a critical role in myogenesis [41, 42].

In somite explants induction of MyoD by Wnt is only observed in the presence of Shh [34]. This requirement for Shh signalling has been confirmed in vivo as loss of MyoD expression following notochord and floorplate removal can be rescued by grafting a Shh soaked bead into the excised region [43]. Analysis of mouse embryos lacking Shh shows this signal also controls expression of both Myf5 and MyoD in the epaxial somite [44].

Shh expression in the developing limb has been primarily analysed in its central role in patterning the anterior-posterior axis [45]. However, in contrast to its role as a positive inducer of MRF expression in epaxial somites, in limb muscles ectopic Shh expression delays MRF expression and maintains proliferative myoblasts, ultimately leading to muscle hypertrophy [46, 47].

2.1.3. BMPs

Bone morphogenetic proteins, members of the TGF-β family [48], are well characterised repressors of myogenic differentiation. BMP4 from the lateral mesoderm regulates formation of the hypaxial somite and represses MyoD expression [49]. In the epaxial myotome BMP signals must be inhibited for myogenesis to proceed and Wnt-1, from the dorsal neural tube, induces expression of noggin, an inhibitor of BMP signalling [50, 51]. The inhibitory effects of Shh in limb myogenesis are also mediated, at least in part, by induction of BMP expression [46].

2.1.4. Notch

Notch signalling can have either positive or negative effects on MRF expression, depending on context. Neural crest cells expressing the Notch ligand Delta migrate past the epaxial somite where they activate Notch in the myoblasts of the dorso-medial lip. This then induces expression of Myf5 and the beginning of myogenic differentiation [52]. In limb muscles Notch signalling does not affect Myf5 expression but does inhibit MyoD induction [53].

2.1.5. FGFs

In vertebrates there are 22 members of the fibroblast growth factor (FGF) family which act via four receptor tyrosine kinases, the FGF receptors [54]. Grafting of FGF4 or FGF8 beads adjacent to somites leads to the loss of expression of MyoD and other myogenic markers [55, 56] but induces the expression of the tendon marker scleraxis. However later in somite development FGF from the myotome induces epithelial to mesenchymal transition and translocation of dermomytomal cells into the central region of the myotome [57], a process known to contribute to the satellite cells of the adult [58]. In limbs FGF4 beads have been reported to downregulate MyoD expression [59] although the receptor through which it is though to signal, FGFR4, is required for limb muscle cell differentiation [60] as expression of a dominant negative form of the receptor leads to decreased MyoD expression.

As is often the case in development the response to signalling events is context dependent and it is becoming clear that there are many variant myogenic programmes which are activated in different muscle groups; uncovering these distinct regulatory mechanisms remains an exciting area of muscle biology.

2.2. Molecular and genomic regulation

The ability of the MRFs to induce muscle specific gene expression means that they, in turn, are tightly regulated as inappropriate expression of MRFs could lead to production of ectopic muscles.

To determine the genomic elements controlling the highly specific expression of Myf5 a series of mice have been generated where reporters, such as LacZ, are expressed under the control of specific regions of the surrounding genome. These have revealed a system of remarkable

complexity where Myf5 is controlled by a combination of promoter and enhancer elements that span 150kb of chromosome. The overall expression pattern of Myf5 is made up of numerous smaller patterns, each with a specific enhancer driving Myf5 expression in a particular subset of muscle precursor cells [61-66]. One particularly striking example of the convergence of mouse genetics and experimental approaches is the finding that that in one of these regions, the early epaxial enhancer, binding sites for both Lef and Gli have been identified [67]; these are the molecules responsible for transducing Wnt and Shh signals that had been previously implicated in MRF induction in somite explant experiments [34, 35]

One intriguing exception to the muscle specific expression of Myf5 is found in some regions of the mouse CNS [68]. The other MRFs are not expressed here and, as there is no muscle present, the role of this neural expression was unclear. More recently it has become clear that a genomic reorganisation in evolution is responsible for this inappropriate expression. However to prevent the activation of muscle specific genes in the nervous system the mRNA transcribed here is not translated and endogenous microRNAs are able to repress the production of Myf5 protein [69].

Together this provides both positive and negative mechanisms for the regulation of Myf5. Once Myf5 is expressed it can then induce expression of the other MRFs which also regulate each other. The exception to this is Myf5 which is not induced either by itself or the other MRFs [70-72]. Because of this the other MRFs do not seem to require such complex regulatory regions and have rather simpler genomic control mechanisms.

MyoD expression is largely regulated by two enhancers, the core enhancer located at -20kb, and the distal regulatory region (DRR) loicated at -4kb. These have been extensively analysed by generating enhancer reporter fusions and mutational analysis in mice [73-79] and birds [80] which have shown that the core enhancer is required for the onset of MyoD expression while the DRR has a more important role in later differentiation. Several factors have been indentified which are required for MyoD transcription including Pax3 which acts in concert with DNMRT and Myf5 [81] Six1 and Six4 [82, 83], Pitx2 [84], Sim2 [85] and Foxo3 [86]. Although Myf5 can activate MyoD it is not required in all cases and MyoD can be induced independently by this array of transcription factors [87].

Myogenin expression can be largely recapitulated with a reporter containing 4kb of upstream sequence [11]. Myogenin expression is regulated, at least in part, by MyoD along other factors, such as NFAT [88], which recruit chromatin remodelling complexes to the myogenin locus [89, 90].

3. Biochemical activity of the MRFs

The MRFs are basic-helix-loop-helix (bHLH) proteins, members of a widespread family of transcription factors found throughout eukaryotes [91]. An alignment of the protein sequences of the four MRFs is shown in Figure 2 with important functional domains highlighted. bHLH proteins are well characterised regulators of differentiation and have been implicated in many developmental systems including ear [92], cardiac [93] and neural

differentiation [94]. bHLH proteins bind specific DNA motifs, known as E boxes, normally as heterodimers in combination with the ubiquitously expressed E12 and E57 proteins [95].

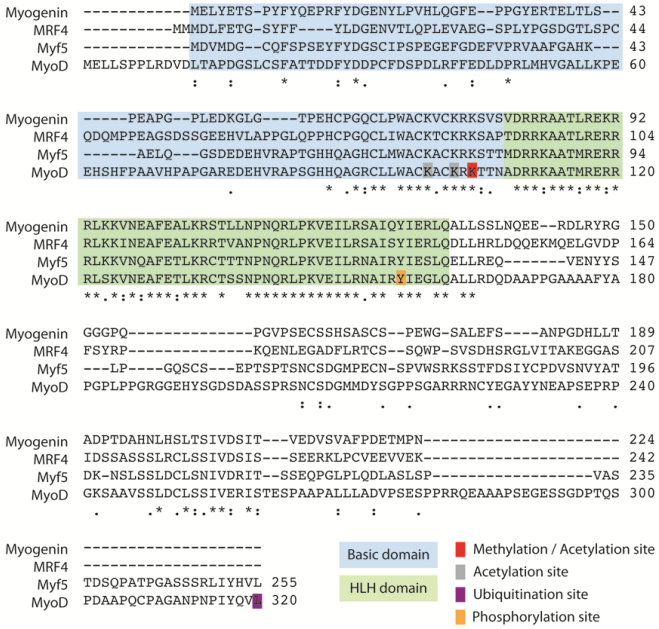

Figure 2. Clustal alignment of human MRF sequences. Basic domain is highlighted in blue, helix-loop-helix domain in green. MyoD methylation / acetylation sites shown in red, acetylation sites in grey, ubiqutination site in purple and phosphorylation site in orange.

Although they have similar biochemical activities in vitro and can bind E boxes in DNA it is clear that there are distinct biochemical activities and functions for the individual MRFs. MyoD and myogenin have been directly compared in their ability to bind to and activate transcription from several muscle specific promoters, such as the chicken myosin light chain, [96, 97] as well as in more global genome binding analysis [98]; it is clear that they bind distinct subsets of promoters and have different sets of target genes. Similar experiments have shown different DNA binding activity of MRF4, MyoD and Mgn [99] while comparison of Myf5 and MyoD activity has mapped part of this differential transcriptional activity to the N and C terminal regions of MyoD which co-operate to give increased transcriptional activation of specific genes which are not activated by Myf5 [100].

An interesting question is how MRF binding to DNA is able to specifically activate muscle gene expression. Recruitment of MyoD to E boxes can be enhanced by the presence of DNA quadruplex structures in promoters [101]; however E boxes are widespread throughout the genome and global analysis of MyoD binding suggests it is able to interact with a large number of these even though they are not associated with muscle specific genes and so do not result in transcriptional activation [102]. Part of the answer to this is that while MRF binding is required for muscle gene expression it is not sufficient and other transcriptional

activators, such as the Six [82, 83] and Pbx proteins [103, 104] are also required at muscle gene promoters to drive expression. However the widespread binding of MyoD may have a broader function and it has been suggested that this can lead to generalised remodelling of the genome in preparation for myogenic differentiation [102]. A similar role has been proposed where MyoD binding is first required at distal enhancers of repressed myogenic genes which have promoter elements inaccesible to transcription factor binding due to their chromatin structure. Interactions between these distal enhancers and more proximal promoters leads to chromatin remodelling at that locus. This opens the promoter and makes it available for MRF binding [105]. It is tempting to speculate that this may be the reason for the pulse of Myf5 expression in paraxial mesoderm prior to somite formation and that this is preparing cells for subsequent inductive events and thus enabling myogenesis.

A recent comparison has also shed light on the specificity of target gene activation by MyoD. Comparison of MyoD binding with a neuronal bHLH protein, NeuroD2, has identified both common and specific E box sequences that these proteins can bind. MyoD specific E boxes are linked to transcription of muscle specific genes while binding to the common E boxes results in broader epigenetic modifications [106].

The activity of MyoD is also regulated by several biochemical modifications and interactions. MyoD is regulated by ubiquitination at its N terminal which targets it for degradation [107, 108]. MyoD is also negatively regulated by methylation which impairs its ability to induce differentiation [109]. MyoD is also acetylated [110] and phosphorylated [111], with both events seeming to enhance MyoD activity. Many of the residues modified in MyoD are conserved across the other MRFs (see figure 2) and it is possible that they are also regulated in this way.

As well as interaction with the E proteins required for transcriptional activity MyoD has also been reported to interact with a wide range of other proteins including c-jun [112], CTCF [113], BAF60c [114], CLP-1 and HDAC at the cyclin D promoter [115], TAZ at the Mgn promoter [116] and β-catenin [117]. MyoD can also interact with cell cycle regulators such as pRB [118] and cdk4 [119] to induce cell cycle withdrawal directly during myogenic differentiation.

This range of interactions shows clearly that the control of MyoD activity is a carefully regulated process and subject to numerous levels of control.

3.1. Targets of MRFs

The biochemical differences in the MRFs contribute directly to their distinct functional roles. Myf5 is able to activate genes required for myogenic commitment while MyoD can also switch on differentiation genes [100]. Similarly MyoD and MRF4 have distinct sets of targets and differentially affect proliferation and differentiation [120].

Myogenin acts downstream of MyoD and is often only able to activate transcription from promoters which have already been bound by MyoD [98]. Myf5, MyoD and Myogenin binding of target sequences is also temporally regulated, providing another mechanism for specificity of target gene activation [121].

One of the best characterised MyoD targets is myogenin. MyoD can bind the myogenin promoter along with Mef2 (another transcription factor and MyoD target gene) [122, 123]. Myogenin, MyoD and Mef2 then co-operate with other transcriptional regulators, such as Six proteins, to activate muscle specific genes such as muscle myosins [96] or muscle specific microRNAs [71, 124] via demethylation of promoter elements [125].

4. Animal models of MRF function

Probably the most widely used animal models to study MRF function are transgenic mice. However knockout animals have shown surprisingly mild effects and mice lacking MyoD [126], Myf5 [127] and MRF4 [128, 129] are all able to develop apparently normal muscle although delays in myogenesis do occur in the limbs of MyoD [130] and somites of Myf5 [127] mutant animals. In contrast mice lacking myogenin have severe muscle defects and die soon after birth [131, 132].

Double knockouts of MyoD and Myf5 were originally reported to lack muscle [133] although subsequently it appears that these mice also lacked MRF4 expression as the targeting of Myf5 had also affected the closely linked MRF4 locus. In MyoD / Myf5 null animals which retain functional MRF4 this gene is able to compensate and initiate myogenesis [134]. Knockout mice have shown that the relationship between the different MRFs is complex and one probable explanation for the functional redundancy of these proteins is that in the absence of one another will be upregulated to substitute for it [126, 128]. The exception to this is myogenin which has a unique, non-redundant function [135] which cannot be compensated for by the other MRFs. Overlapping roles for MRFs are also demonstrated in mice lacking MyoD and MRF4 which have severe muscle defects [136]. It is also apparent that Myf5 alone is not sufficient to support myogenic differentiation as in mice lacking the other MRFs myogenesis is initiated but not maintained [137]. This specificity of individual MRF function has also been demonstrated in other animal models such as Xenopus [138], zebrafish [139] and chickens [70, 71] although it is striking that some specific functions of MRFs have changed during evolution. An example of this is the regulation of the muscle specific microRNA miR-206 which appears to have different requirements for MRF expression in mice, chickens and fish [70, 71, 140].

The role of Myf5 has been further examined by the production of transgenic mice which express diptheria toxin under the control of Myf5, thus ablating all Myf5 expressing cells in the embryo. Fascinatingly these mice develop morphologically normal muscle [141, 142], suggesting that a Myf5 independent population of myoblasts are present and can expand to fill the niche left by loss of Myf5 expressing cells. This correlates well with data showing that distinct regulation of MyoD and Myf5 defines different subsets of cells based on reporter gene expression [16].

5. Summary

Although great strides have been made in understanding the MRFs at biochemical, genomic and whole animal levels there remain significant unanswered questions. Among these is

issue of what are target genes of each MRF in vivo and how do they differ in their activity in different muscle types. Understanding the answers to these questions will provide key insights which will directly influence both basic science and regenerative medicine.

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Chapter 3

Retinoid X Receptor Signalling in the Specification of Skeletal Muscle Lineage

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Additional information is available at the end of the chapter

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1. Introduction

Pluripotent stem cells have the capacity to develop into different cell lineages, and can be promoted into skeletal muscle lineage through the use of small molecule inducers. Retinoic acid (RA) signaling through the retinoic acid receptor (RAR) and retinoid X receptor (RXR), is important for embryonic development, and is able to enhance myogenic differentiation *in vitro* if used in combination with other small molecule inducers. Nevertheless, it only yields moderate results in promoting the differentiation of embryonic stem (ES) cells into skeletal myocytes. RXR is also known to be essential for embryonic development, but it is generally considered to act as a silent partner for other nuclear receptors such as RAR. We recently discovered that RXR selective ligand efficiently induces myogenic differentiation in mouse ES cells which respond poorly to RA. In addition, myogenic differentiation, enhanced by the RXR ligand, is mediated through a RAR independent mechanism, and recapitulates closely the sequential events observed *in vivo.* Since ES cell differentiation represents the properties of early developing embryo, efficiently generating skeletal myocytes with RXR selective ligand provides means to further scrutinize signaling pathways in skeletal myogenesis, in view of developing cell-based therapies for skeletal muscle-related diseases. In this chapter, we attempt to provide an in-depth analysis of recent research findings and the current stage of knowledge in the field of skeletal myogenesis.

2. The retinoid X and retinoic acid receptors

RXR belongs to the nuclear hormone receptor superfamily, such as steroid hormone, thyroid hormone, vitamin D receptors, and nuclear receptors including RAR, PPAR, LXR and PXR (Szanto *et al*., 2004). It is a very unique protein with the ability to form heterodimers with one third of the 48 other nuclear receptors (Mangelsdorf *et al*., 1995) giving it the potential to converge a large array of signaling pathways. The RXR can form homodimers, permissive

heterodimers, and non-permissive heterodimers in a ligand-dependent or -independent manner (Tanaka and De Luca, 2009). When RXR forms homodimers or permissive heterodimers (with PPAR, LXR, PXR etc.), it is amenable to RXR ligand-dependant activation since the activation domain of the partner receptor is placed in proximity to RXR helixes. Once RXR is activated by the ligand, conformational changes cause direct stabilization of the activation domain of its partner (Gampe, Jr. *et al.*, 2000b). When RXR forms non-permissive heterodimers (with RAR, VDR, TR etc.), it is not activated by ligand, as the binding of the partner receptor to RXR allosterically inhibits it (Kurokawa *et al.*, 1994; Tanaka *et al.*, 2009). Furthermore, the activation domain of the partner is not located in proximity to ligand activated residues in the RXR interface (Bourguet *et al.*, 2000; Gampe, Jr. *et al.*, 2000b).

2.1. DNA binding

The receptor dimers of RXR and its partner, constitutively bind to specific DNA response elements in the promoters or enhancers of the genes they govern. DNA binding specificity is determined by the number of spacer nucleotides present between two direct repeats of the canonical binding sequence 5′-PuGGTCA (Leid *et al.*, 1992; Umesono and Evans, 1989). The RXR/RAR heterodimers bind to the retinoic acid response element (RARE) with a consensus half site separated by 2 or 5 nucleotides (DR2 or DR5), whereas the RXR homodimers bind to the retinoid X response element (RXRE) separated by only one nucleotide (DR1) (Tanaka *et al.*, 2009) (Figure 1). Selective response element recognition is due to a short sequence (the P box) located at the C-terminal base of the N-terminal C1 finger of the DNA binding domain (DBD) which interacts with the binding motif, and also due to a weak dimerization function which encompasses the N-terminal base of the CII finger (D-box) of the DBD (Danielsen *et al.*, 1989; Green *et al.*, 1988; Kumar and Chambon, 1988; Luisi *et al.*, 1991; Mader *et al.*, 1989; Umesono *et al.*, 1989). While RXR/RAR heterodimers bind more effectively to the RAREs than RXR homodimers, RXRs homodimers can bind RXREs with high affinity (Zhang *et al.*, 1992). RAREs can overlap with RXREs, and since RXR/RAR heterodimers bind with a higher affinity than RXR homodimers, (Tanaka *et al.*, 2009), this may interfere with RXR signaling.

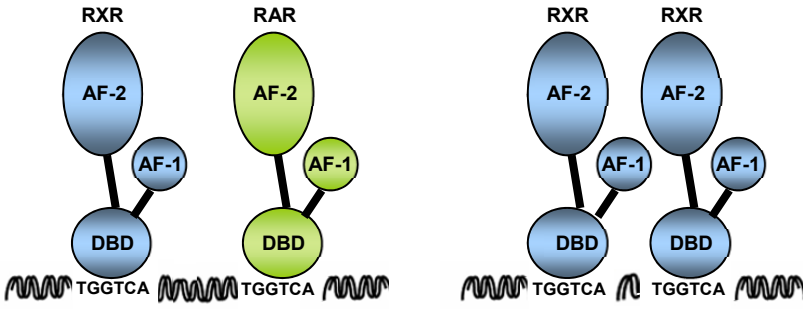

Figure 1. The Binding of RXR/RAR Heterodimer and RXR Homodimer to DNA. RXR/RAR heterodimers (left) and RXR homodimers (right) bind via the DNA binding domain to two direct repeats of the canonical binding sequence 5′-PuGGTCA separated by 2 or 5 nucleotides, or 1 nucleotide respectively.

2.2. Ligands of RXR and RAR

While RXR and RAR constitutively bind to DNA, they require agonist binding to activate gene transcription. Several endogenous ligands are well characterized and many synthetic ligands have been developed.

RA, the active derivative of vitamin A, can exist as two isoforms: all-trans RA and 9-cis RA. RAR bind and are activated by all-trans RA as well as its 9-cis isomer, while the RXR bind and are activated only by 9-cis RA (Ricaud *et al.*, 2005). However, due to the considerable difficulty of detecting 9-cis RA endogenously in embryos or in adult tissue (Niederreither and Dolle, 2008), there has been debate about the *in vivo* role of activated RXR, and has led to the belief that RXR serves only to orient and position the heterodimers properly on the DNA (Perlmann and Jansson, 1995; Willy *et al.*, 1995; Willy and Mangelsdorf, 1997)

In the last two decades, a wide range of RXR selective compounds has been engineered. The synthetic RXR ligands can act as agonists and activate both homodimers and permissive heterodimers. Conversely, they can also act antagonistically of homodimers, as is the case for the synthetic ligand LG100754, and promote only the activation of non-permissive heterodimers (Lala *et al.*, 1996). Bexarotene (LGD1069) is a synthetic RXR selective compound used in the treatment of cancer. It is unable to transactivate the RXR-RAR heterodimer (Lehmann *et al.*, 1992) and will not activate RARs (Nau *et al.*, 1999).

There are conflicting interpretations of RXR participation in the activation of RXR/RAR heterodimers. Some studies demonstrate that allosteric inhibition of RXR in the RXR/RAR heterodimer only occurs when the RAR is unliganded and that this inhibition is relieved once RAR is liganded (Forman *et al.*, 1995; Lala *et al.*, 1996). Other reports indicate that both receptors bind their ligands independently and that their effects are additive (Kersten *et al.*, 1995). The discrepancy between varying reports can possibly be reconciled by the fact that different ligands interact with distinct side chains in the ligand binding domain and thus mediate differential activation of the receptor complex. The exact response is therefore highly dependent on the identity of the ligand and cannot simply be classified as agonistic versus antagonistic. Although RXR can engage in ligand binding when RAR is ligand occupied and/or if a suitable synthetic ligand is present (Chen *et al.*, 1996; Kersten *et al.*, 1996; Lala *et al.*, 1996; Minucci *et al.*, 1997; Roy *et al.*, 1995), bexarotene is unable to transactivate the RXR/RAR heterodimer (Lehmann *et al.*, 1992). In fact, bexarotene has been reported to reduce interactions between RXRs and RARs whereas ligand such as 9-cis increases the binding of RXRs to RARs (Dong and Noy, 1998).

All-trans RA does not bind RXR (Mangelsdorf *et al.*, 1992), and more importantly, although all-trans RA has the ability to isomerize to 9-cis RA, pharmacological doses of all-trans RA are required to generate enough 9-cis to activate the RXRs (Mic *et al.*, 2003). Optimal enhancement of skeletal myogenic differentiation requires low concentrations of all-trans RA. Thus, all-trans RA isomerization is simply not a feasible explanation to the similar enhancement of myogenic differentiation by RA and bexarotene observed in P19 stem cells

(Le May *et al.*, 2011). Finally, while RA metabolites, such as 4-oxo-RA, were originally believed to play a role in RA signaling, they have more recently been shown as physiologically not required (Niederreither *et al.*, 2002; Pijnappel *et al.*, 1993).

2.3. The interaction of RXR and RAR with their cofactors

In response to ligand activation, RXR and RAR bind co-activators and the respective binding of cofactors again depends on the identity of the ligand. Agonist binding induces large conformational changes within the receptor causing helix 11 and 12 (the AF-2 domain) to close the lid of the ligand binding pocket and generate high affinity co-activator binding sites. This charged surface has a high affinity for a specific amino acid motif, LXXLL, which mediates the binding of co-activators to nuclear receptors (Westin *et al.*, 1998). Alternatively, if an antagonist or partial agonist binds, helix 12 is repositioned to an adjacent groove on the LBD and a charged surface that favors the co-repressor binding motif is formed (Perissi *et al.*, 1999).

Co-activators, as their name implies, have the ability to activate transcription and interact with the basal transcriptional machinery, bridge and direct the assembly of transcriptional pre-initiation complexes, and induce chromatin remodeling (Rosenfeld *et al.*, 2006; Bastien and Rochette-Egly, 2004). Co-activators such as p300, CREB Binding Protein (CBP), and p300/CBP-Associated Factor (PCAF) can all act as histone acetyltransferases (HATs) (Niederreither *et al.*, 2008; Ogryzko *et al.*, 1996) and form large multimolecular complexes.

Interestingly, co-activators p300 and CBP are also able to acetylate proteins other than histones, such as transcription factors (Gu and Roeder, 1997; Li *et al*. 1998; Li *et al*. 1999). CBP and p300 are heavily autoacetylated and upon recruitment to the receptors, can acetylate more of themselves in an intermolecular fashion (Karanam *et al.*, 2006). In addition to this, they have the ability to recruit PCAF (Yang *et al.*, 1996), a coactivator involved in myogenesis. p300 influences RXR activity as RXR are subjects for p300 acetylation, which promotes their binding to RXRE and increases their transcriptional activity as well (Zhao *et al.*, 2007). Co-activators play crucial roles in gene activation, however, those recruited by particular RXR dimers at specific genetic loci in response to ligand have yet to be identified.

Alternatively, in the absence of ligand, the co-repressors, such as the nuclear receptor corepressor (NCoR) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) family, bind and recruit a mulitprotein complex containing the histone deacetylase HDAC3 (Guenther *et al.*, 2000; Li *et al.*, 2000). The recruited histone methyl-transferases and histone deacetylases stabilize the nucleosome structure so that the DNA is inaccessible for transcription (Niederreither *et al.*, 2008).

When RXR forms permissive heterodimers (i.e.: RXR/PPAR), neither receptor binds the co-repressors under normal circumstances (DiRenzo *et al.*, 1997). Ligand binding to one receptor recruits the co-activators and although the other receptor may be unliganded, the high local concentration of bound co-activators favor the docking of the second LXXLL motif with the co-activator binding sites of the other receptor. If ligand is present for both

receptors of the permissive heterodimer, they can synergistically recruit co-activators (Ahuja *et al.*, 2003).

Non-permissive heterodimers (i.e.: RXR/RAR) do bind co-repressors and this binding to unliganded RXR and its partner is stabilized by both receptors. Transactivation requires ligand binding to the RXR partner (i.e.: RAR) to convert it into the agonist conformation, displace co-repressors, and recruit co-activators (Zhang *et al.*, 1999; Vivat *et al.*, 1997). As with permissive heterodimers, synergistic recruitment of co-activators occurs when ligands are present for both receptors (Ahuja *et al.*, 2003).

2.4. RXR and RAR in development

Gene mutation studies have determined that both RXR and RAR are essential for proper development, and delineated roles and tissue expression patterns for the different isoforms of the two receptors (α, β, and γ). The different RAR and RXR isotypes are encoded by different genes and their isoforms differ in their NH2-terminal regions which are generated by differential promoter usage and alternative splicing (Chiba *et al.*, 1997). While RXR-α null embryos show defects in RXR/PPARγ (Peroxisome Proliferator Activated Receptor) signaling, the RARs appear to be the most important partners for RXRs (Ahuja *et al.*, 2003). RXR/RAR non-permissive heterodimers have been extensively studied in the context of development.

During development, RXR-α and β are ubiquitously expressed with the highest levels of RXR-α present in the liver, heart, intestines, kidney, spleen, placenta, and the epidermis (Ahuja *et al.*, 2003; Pratt *et al.*, 1998). RXR-γ is expressed in all developing skeletal and cardiac muscles, the anterior pituitary, and the brain. The expression of RXR isoforms is tissue specific and often overlaps, yet occasionally certain isoforms are uniquely expressed.(Mangelsdorf *et al.*, 1992) RXR-α is the primary isoform and supports the activity of all three RARs. Furthermore, RXR-α may be important in the expression of RXRγ since the RXRγ gene contains a RXRE (Barger and Kelly, 1997).

Studies with mice lacking expression of RXR-α have found that these mice die in utero as a result of hypoplastic myocardium (Kastner *et al.*, 1994; Sucov *et al.*, 1994) and RXR-α null mutations exhibit growth retardation, webbed digits (Mark *et al.*, 2006) and defects in the chorioallantoic placenta (Sapin *et al.*, 1997). Loss of RXR-β and RXR-γ is not as severe since they can be compensated for by RXR-α (Tanaka *et al.*, 2009), which may explain why the RXR$\gamma^{-/-}$ mouse mutants are viable and have no muscular defects even in compound mutant combinations (Dolle, 2009).

Similarly, animals lacking RAR-α or RAR-γ result in postpartum lethality (Lohnes *et al.*, 1993). In RAR knock-out studies where two RARs are deleted, the mutants display a spectrum of defects that resemble vitamin A deficiency syndrome (Lohnes *et al.*, 1993; Lufkin *et al.*, 1993) and the function of the residual RAR is highly dependent on RXR-α (Ahuja *et al.*, 2003).

Even in normal development, the RARs are highly dependent on the RXRs. Homodimer formation of RARs is energetically unfavored, because of the limited contact between the interfaces. Pairing with RXR creates an extended area of intermolecular contact that stabilizes the heterodimer formation. This substantially larger surface area and consequent stability, results in the preferential formation of RXR/RAR heterodimers (Bourguet *et al.*, 2000; Gampe, Jr. *et al*., 2000a).

3. Skeletal myogenesis

Skeletal muscle forms in the embryo from paraxial mesoderm, which segments into somites on either side of the neural tube and notochord (Christ and Ordahl, 1995). Extracellular signals from surrounding tissues play a significant role in muscle development. These signals include members of the Wnt family, specifically Wnt1 and Wnt7a secreted from the neural tube and surface ectoderm (Cossu and Borello, 1999), Sonic Hedge Hog (Shh) secreted by notochord and floor plate cells and which acts in conjunction with Wnt1 (Cossu *et al.*, 1999), bone morphogenetic protein4 (BMP4) secreted by the lateral plate mesoderm cells (Borycki *et al.*, 1999; Dietrich *et al.*, 1998; Munsterberg *et al.*, 1995; Pourquie *et al.*, 1996; Tajbakhsh *et al.*, 1998), and RA which is under tight regulatory control for its synthesis, degradation, and transport (Rohwedel *et al.*, 1999). These act on downstream targets such as HOX genes, which controls specification of the body axis (Rohwedel *et al.*, 1999), Brachyury T, a protein required for posterior mesoderm and notochord differentiation (Skerjanc, 1999), and the myogenic regulatory factors (MRFs) including Myf5, MyoD, myogenin, and Mrf4 which are required for the commitment and maturation of skeletal muscle (Cossu *et al.*, 1999; Rohwedel *et al.*, 1999; Skerjanc, 1999).

3.1. Myogenic regulatory factors and their cofactors

The formation of myoblasts from myogenic progenitors and their successive cell cycle arrest and differentiation into mature skeletal muscle involves two key families of transcription factors. The MyoD family of basic Helix-Loop-Helix (bHLH) proteins which includes the four master transcriptional regulators (also referred to as MRFs): Myf5, MyoD, myogenin, and Mrf4 (Arnold and Braun, 2000; Braun *et al.*, 1989; Braun *et al.*, 1990; Davis *et al.*, 1987; Edmondson and Olson, 1990; Froeschle *et al.*, 1998) and the myocyte enhancer factor 2 (MEF2) family of MADS-box transcription factors which includes MEF2A, -B, -C, and –D (Naya and Olson, 1999).

Myf5 and MyoD are involved in skeletal muscle specification and commitment and have the capacity of remodeling chromatin and opening gene loci that participate in further muscle differentiation (Bergstrom and Tapscott, 2001), whereas terminal differentiation is governed by myogenin and MRF4. Each MRF is sufficient to dominantly induce myogenesis when introduced into a variety of non-muscle cells (Olson, 1990; Weintraub, 1993), and ectopic expression of MyoD can inhibit cell cycle before the S phase independently of its DNA

binding and the induction of myogenic differentiation (Crescenzi *et al.*, 1990; Sorrentino *et al.*, 1990).

Members of the MEF2 family alone are not sufficient to induce myogenesis, however the ability of the MRFs to convert cells is reliant on the function of the MEF2 family. MEF2 proteins bind as homodimers and heterodimers to the consensus sequence YTA(A/T)4TAR found in the promoter region of nearly every known muscle-specific gene (Black and Olson, 1998), and together with the myogenic bHLH proteins, synergistically activate the transcription of myogenic genes. Unlike the MRFs, MEF2 genes are also expressed outside the skeletal muscle lineage in tissues such as cardiac and smooth muscle (Black *et al.*, 1998; Edmondson *et al.*, 1994; Leifer *et al.*, 1993; Lyons *et al.*, 1995).

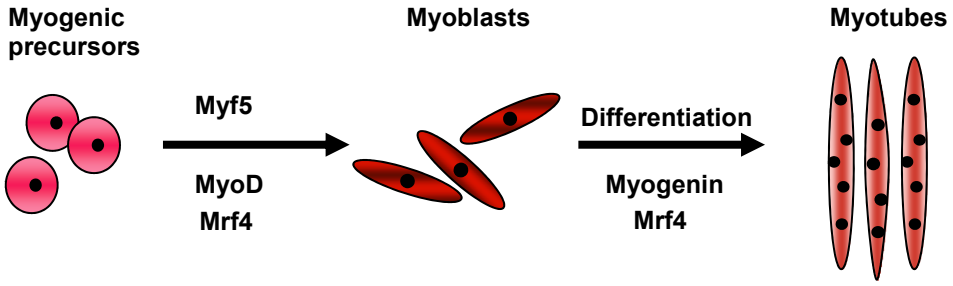

Figure 2. Involvement of Myogenic Regulatory Factors in Myogenesis. Myf5 and MyoD are involved in specification and commitment of muscle progenitors into skeletal muscle lineage. Mrf4 also plays a role as a determination gene in addition to directing terminal differentiation along with myogenin.

The bHLH domain of the MRFs is responsible for DNA binding and for dimerization with the ubiquitously expressed bHLH E protein (Hu *et al.*, 1992; Murre *et al.*, 1989; Parker *et al.*, 2006). The resulting myogenic bHLH-E heterodimers bind to DNA at the consensus sequences known as an E-box (CANNTG), specific DNA motifs present at muscle gene enhancers and/or promoters, where they regulate gene expression (Sartorelli and Caretti, 2005). These genes include cytoskeletal, sarcomeric, metabolic, and cell signaling proteins (Angus *et al.*, 2001; Gramolini and Jasmin, 1999; Kraner *et al.*, 1999; Li and Capetanaki, 1993; Lin *et al.*, 1991; Marsh *et al.*, 1998; Shield *et al.*, 1996; Simon and Burden, 1993; Wheeler *et al.*, 1999). A requirement for the MyoD family of transcription factors in this combinatorial complex is demonstrated by the fact that the E protein homodimers bind to the same DNA sequences as the MyoD-E protein heterodimers, yet only the MyoD-E protein complex can cooperate with MEF2 factors (Naya *et al.*, 1999). Furthermore, the MRFs and MEF2 factors activate and repress each others transcription in a complex network (Arnold and Winter, 1998; Bergstrom *et al.*, 2002; Cserjesi and Olson, 1991; Olson and Klein, 1994; Wong *et al.*, 1994). For example, expression of myogenin requires MEF2, while myogenin activates the expression of MEF2 independently of other skeletal gene products (Cserjesi *et al.*, 1991; Ridgeway *et al.*, 2000). Similarly, MRFs can positively regulate their own transcription and

transcription of each other, creating positive auto- and cross-regulatory loops (Braun *et al.*, 1989; Thayer *et al.*, 1989)

3.2. Roles of meox and pax in the specification of myogenic progenitors

Signals from surrounding tissues activate the premyogenic program, and result in the expression of transcription factors such as Pax3, and Meox1/2 that specify cells into the skeletal muscle lineage and mediate the induction of the MRFs (McDermott *et al.*, 2005; Petropoulos and Skerjanc, 2002; Petropoulos *et al.*, 2004; Ridgeway and Skerjanc, 2001; Williams and Ordahl, 1994).

Pax3, a transcription factor with homeo and paired domain motifs, is thought to be activated by Wnt6a (Fan *et al.*, 1997) and is responsible for both delamination and migration of muscle progenitors to the limb bud (Tajbakhsh *et al.*, 1997). Pax3 is initially expressed throughout the somite before becoming restricted to the dermomyotome and subsequently the migratory muscle progenitor cells (Goulding *et al.*, 1994; Williams *et al.*, 1994). The importance of Pax3 in the delamination and migration of muscle progenitor cells is highlighted by the fact that mice which are Pax3 null have severe muscle loss (Alvares *et al.*, 2003; Bladt *et al.*, 1995; Dietrich *et al.*, 1999; Epstein *et al.*, 1996; Grifone *et al.*, 2005).

Pax3 directly regulates the expression of Myf5 through the limb bud enhancer of Myf5 gene (Bajard *et al.*, 2006) and acts with Myf5, upstream of MyoD which cannot be properly expressed in the Pax3/Myf5 double knockout (Tajbakhsh *et al.*, 1997). It is when the migrating cells reach the limb bud that they begin to express Myf5 and MyoD, and it is both before and after activation of these genes that the cells undergo extensive proliferation (Buckingham *et al.*, 2003; Tajbakhsh and Buckingham, 1994). Pax3, along with additional factors such as Myf5, c-met, Msx1 and the fibroblast growth factor (FGF) family of receptors promote myoblast proliferation. Proliferation is arrested by inhibitory signals which promote differentiation by inducing cell cycle arrest proteins such as MyoD (Alric *et al.*, 1998).

Meox1 and Meox2 are closely related homeobox genes with mesoderm and mesenchyme specific expression during mouse development (Candia *et al.*, 1992). Meox1 is expressed in the dermomyotome whereas after delamination and migration to the limb bud, Meox2 is predominantly expressed (Candia *et al.*, 1992; Candia and Wright, 1996). In Meox2 deficient limb buds, Pax3 and Myf5 are downregulated and mice homozygous for a null mutation in Meox2 have defects in limb muscle differentiation resulting in an overall reduction in muscle mass and absence of specific muscles (Mankoo *et al.*, 1999). It is only the compound mutant embryos of $Meox1^{-/-}$; $Meox2^{-/-}$ that display a dramatic phenotype associated with disrupted somite development. In these embryos, the axial skeleton fails to develop and most skeletal muscles are absent or reduced in size (Mankoo *et al.*, 2003). Interestingly, in cell cultures, overexpression of Meox1 does not induce myogenesis and while a dominant negative Meox1 has been shown to downregulate Pax3 and Gli2 expression and inhibit myogenesis in the P19 stem cells (Petropoulos *et al.*, 2004), Meox1 mutant mice exhibit mild defects in sclerotome-derived vertebral and rib bones (Mankoo *et al.*, 2003) rather than showing any overt muscle defects.

3.3. Roles of histone acetyltransferases in myogenic expression

Not only are extracellular signals crucial for the proper induction of MRFs, but intracellular prompts involving acetyletransferases play a fundamental role as well. CBP and p300 are required for growth arrest and apoptosis (Vo and Goodman, 2001), and along with PCAF are required for terminal differentiation of myoblasts and transactivation of muscle specific promoters such as myosin heavy chain (MHC) and muscle creatine kinase (MCK) (Eckner *et al.*, 1996; Polesskaya *et al.*, 2001; Puri *et al.*, 1997a; Puri *et al.*, 1997b; Yuan *et al.*, 1996). Embryonic stem cells lacking p300 or its HAT activity are strongly impaired in their ability to activate Myf5 and MyoD (Roth *et al.*, 2003). When properly expressed, Myf5 and MyoD, in cooperation with MEF2 transcription factors and with p300 and CBP, mediate the activation of the secondary MRFs, myogenin and Mrf4. p300 has been shown to bind directly to MyoD (Sartorelli *et al.*, 1997; Yuan *et al.*, 1996), and both p300 and PCAF play a critical role in the maximal MyoD dependant transactivation; p300 acetylates histones H3 and H4 and recruits PCAF to the promoter whereas PCAF acetylates MyoD to enhance transcription initiation, increase its affinity for DNA binding, and facilitate heterodimer formation with E proteins (Dilworth *et al.*, 2004; Puri *et al.*, 1997a; Sartorelli *et al.*, 1999). However, MyoD has also been found to be acetylated in proliferating myoblasts where it is inactive, therefore further mechanisms besides simply acetylation are required for MyoD activation (Polesskaya *et al.*, 2000).

4. Impact of extracellular cues on MRF expression

Ligands of RAR and RXR play important roles in the activation of myogenesis and this activation is highly dependent on the identity of the ligand. RA is required for proper somite formation (Maden *et al.*, 1996; Maden *et al.*, 2000; Niederreither *et al.*, 1999), induction of specification genes Meox1, Meox2, and Pax3, and counteracts inhibitory signals such as BMP4 (Kennedy *et al.*, 2009). RA signaling intersects with that of BMP4, as BMP4 and RA function antagonistically and have the capacity to counteract each other's inhibition of entry into skeletal and cardiac muscle lineages (Kennedy *et al.*, 2009). Low concentrations of RA can regulate the levels of Myf5 implying the existence of a RARE in the Myf5 regulatory region (Carnac *et al.*, 1993). RA also enhances MyoD and myogenin expression (Carnac *et al.*, 1993), and RA receptors and MyoD have been found to upregulate each other's transcriptional activity; their transcriptional co-activation requires a RA receptor-MyoD complex that binds to MyoD DNA binding sites in muscle cells (Froeschle *et al.*, 1998). RA is capable of inhibiting proliferation of myoblasts through inducing cell cycle arrest proteins (Alric *et al.*, 1998) and in vitamin A deficient embryos, myogenin is downregulated (Maden *et al.*, 2000) providing a link between RA and myoblast maturation.

RA and bexarotene are both capable of inducing skeletal myogenesis in the P19 stem cells, however, they do so through differential activation of crucial specification genes (Le May *et al.*, 2011). Bexarotene primarily activates Meox1 while RA mainly activates Pax3. Nontheless, both ligands are equally capable of inducing later target genes such as MyoD and myogenin. Alternatively, only bexarotene is capable of inducing myogenesis in ES cells

to a significant level (Le May *et al.*, 2011). Furthermore, treatment of these cells with bexarotene gives long, mature, multinucleated myofibers.

4.1. Stem cell as a model for study of myogenic differentiation

It is highly advantageous to use stem cell tissue cultures to study the importance of specification genes in a controlled environment to understand their relationship with each other and their regulation by extracellular signaling molecules. Specification factors exist in a very complex relationship and have the ability to autoregulate and cross-regulate one another (Petropoulos *et al.*, 2004).

In P19 stem cell cultures, Pax3 overexpression can induce Meox1 but is unable to activate Gli2 and a dominant negative Pax3 mutation does not affect Gli2 levels. Conversely, Gli2, which also has the ability to upregulate Meox1, can upregulate Pax3 while the dominant negative Gli2 P19 cells downregulate Meox1, Pax3, and MyoD expression and inhibits myogenesis. Lastly, Meox1 can activate the expression of Gli2 but overexpression of this protein is insufficient to induce Pax3 or skeletal myogenesis (Petropoulos *et al.*, 2004). The ability of each of these factors to induce each other, or, in their absence, completely abolish myogenesis underlines the importance of these factors in the specification process.

Wnt signaling via β-catenin is also essential and sufficient for the induction of specification factors Pax3, Meox1, and Gli2 and in P19 stem cells, a dominant negative β-catenin inhibits Pax3, Gli2, Meox1 and MyoD expression and abolishes myogenesis (Petropoulos *et al.*, 2002). This is not surprising since mutations of either Gli2, Meox1, or Pax3 in these cells will abrogate myogenesis (Petropoulos *et al.*, 2004). Pax3 expression is essential and sufficient for the expression of the transcription factor Six1 and the induction of skeletal myogenesis (Ridgeway *et al.*, 2001). Its overexpression induces Myf-5, MyoD, and myogenin expression (Maroto *et al.*, 1997) whereas a dominant negative Pax3 in P19 cells results in a loss of MyoD and myogenin expression and subsequent myogenesis (Ridgeway *et al.*, 2001).

4.2. Significance of a separate RXR signaling pathway

The importance of a separate, rexinoid signaling pathway in skeletal muscle development non-overlapping with RA signal transduction is demonstrated by the fact that an RXR selective ligand, bexarotene effectively enhances skeletal myogenesis in mouse ES cells that respond poorly to RA (Le May *et al.*, 2011). This difference in the two signaling pathways stems from differential activation of very early genes involved in crucial lineage specification, although both bexarotene and RA are dependent on functional β-catenin signaling (Le May *et al.*, 2011). It is intriguing that a cell type such as ES cells, that has thus far been relatively resistant to RA-induced skeletal muscle differentiation develops so well in the presence of an RXR selective ligand, especially considering these cells do not posse the necessary machinery to synthesize 9-cis RA, the purported endogenous ligand (Chen and Khillan, 2010). It appears that P19 cells have the ability to differentiate by both retinoid and rexinoid signaling instigated pathways while ES cells respond well only to rexinoid

mediated pathways. Similarly, RAC65 cells resistant to RA-induced skeletal muscle and neuronal conversion (Costa and McBurney, 1996) demonstrate efficient skeletal differentiation when treated with RXR selective ligand (Le May *et al.*, 2011). The ability of rexinoid to bypass the dominant negative RAR inhibition in RAC65 cells is not unique to skeletal muscle and has also been documented for neuronal differentiation as well (Yokota and Ohkubo, 1996). Finally, RXR is able to activate target genes involved in RA signaling that cannot be induced by RARs as is the case with the response element in the CRBPII (Cellular Retinol Binding Protein Type II) gene which contains a DR1, underscoring the possibility of RXR/RXR and RXR/RAR independent pathways (Mangelsdorf *et al.*, 1991).

It remains to be determined which specific co-activators are recruited by RXR in the enhancers or promoters of target genes during skeletal myogenesis. RXR homodimers or RXR permissive heterodimers might recruit a separate set of co-activators and therefore differentially control gene expression. It could be that the unique ability of bexarotene versus RA to control the transcription factor's interactions with co-activators is the method by which distinct and even competing signaling pathways can be distinguished.

4.3. Unsaturated fatty acids activate RXR

The physiological significance of 9-cis RA signaling is debated due to a lack of consensus on its existence in the developing embryo. However, the enzymes that contribute to its biosynthesis are well documented (Mertz *et al.*, 1997; Romert *et al.*, 1998) in addition to its ability to induce the formation of homodimers that bind to DR1 sequences (Zhang *et al.*, 1992). The lack of a known ligand is hardly reason to exclude RXR as physiologically significant *in vivo* and a major factor that supports the presence of an active endogenous ligand is the fact that RXR tetramers cannot dissociate without agonist binding.

Studies using RXR ligand-detector mice have identified specific regions of the spinal cord as major sites of endogenous rexinoid production and classify naturally occurring poly-unsaturated fatty acids, including docosahexaenoic acid (DHA) as a major endogenous ligand for RXR in the mouse brain (Ahuja *et al.*, 2003; de Urquiza *et al.*, 2000). When characterized in the ligand binding domain of RXR-α, DHA has a significantly higher number of ligand-protein contacts than 9-cis and certain synthetic ligands and also has the ability to activate RXR homodimers as well as synergistically activate the RXR-RAR heterodimers in combination with all-trans RA (Lengqvist *et al.*, 2004). It remains to be determined if this ligand is functional in all tissues or whether there are other yet undiscovered ligands. Presently, additional unsaturated fatty acids, including docosapentaenoic, arachidonic, and oleic acids, also have been found to bind and activate RXR, suggesting that this ability is not exclusive for DHA. Irrespective of whether an endogenous RXR ligand does indeed exist, the ability to control cell growth and differentiation through targeting RXR with highly selective ligands confers *many* therapeutic applications to this unique receptor.

5. Therapeutic potentials of rexinoids

It is unknown whether RXR homodimer or RXR permissive heterodimer signaling is the main mechanism governing skeletal muscle differentiation. Regardless, controlling cell processes using RXR selective ligands underlines the fact that two distinct and possibly overlapping pathways exist. Moreover, RAR-independent rexinoid signaling provides another route of achieving cell cycle arrest and differentiation when RA signaling is aberrant, a situation frequently seen in cancer where differentiation often appears to result in loss of a malignant phenotype (Gokhale *et al.*, 2000).

RXR-α overexpression sensitizes tumors to rexinoid-induced anti-growth effects, cellular differentiation, decreased cell proliferation, apoptosis of some type of cancer cells, and prevention of angiogenesis and metastasis (Qu and Tang, 2010). Bexarotene, has been approved by the FDA for use in the treatment of refractory or persistent cutaneous T-cell lymphoma and has the ability to reduce tumor development in several other cancers (Duvic *et al.*, 2001; Wu *et al.*, 2002). However, the use of this compound in the treatment of lung and breast carcinomas has yielded disappointing results (Tanaka *et al.*, 2009) demonstrating our lack of understanding of the molecular mechanisms underlying rexinoid-induced antitumor effects and RXR-induced multi-pathway activation.

One of the reasons rexinoids seem such promising chemotherapeutic compounds compared to retinoids, is that retinoids have numerous side effects which severely limit the dosage and efficacy while rexinoids display mild toxicity. Furthermore, RXR expression is rarely lost in human tumors whereas RAR expression is frequently lost or reduced in various cancers (Sun and Lotan, 2002; Umesono *et al.*, 1989). Since p53 abnormalities are reported in more than 50% of human cancers, and p21 is rarely mutated (Shiohara *et al.*, 1994; Tanaka *et al.*, 2007), RXR mediated induction of p21 is a promising therapeutic target for these cancers. The study of myogenic differentiation may provide some answers to new target genes as the development and progression of cancer involves aberrations in the same mechanisms that regulate cell differentiation during embryogenesis. It remains to be revealed which other genes can also be targeted by rexinoids and which specific interactions take place that we can study and apply to our development of more potent and effective therapeutics.

Pluripotent stem cells closely simulate embryonic development and present a model system with which to dissect signaling pathways of target receptors in controlled environments. They hold a tremendous potential for cell-based therapies through their capacity to grow and regenerate new tissues. Many diseases including muscular dystrophies, cancer, AIDS, and even normal conditions such as aging show prominent muscle loss that would benefit enormously from regenerative cell-based therapies. However, our ability to use stem cells in muscle-wasting disorders has been limited due to the low rate of myogenic differentiation in ES cell cultures and the difficulty in identifying and isolating progenitor cells. To harvest the full potential of these cells in therapies, it is imperative that we find small molecule inducers capable of efficiently directing stem cells into skeletal muscle lineage. Attempts at using RA in ES cell cultures have thus far yielded disappointing results; however, the ability of

rexinoids to induce these cells has not yet been fully explored. Understanding the myogenic pathway *in vivo* as well as deciphering differentiation cues to culture pure populations of myogenic progenitors will prove a vital tool in the treatment of such devastating diseases.

6. Conclusion

RXR selective ligand is an effective inducer of skeletal myogenesis not only in the P19 pluripotent stem cells, but also in the mouse ES cells which have thus far been relatively resistant to RA induction. RXR specific signaling plays an important role in this process through a separate RAR-independent mediated pathway. It appears that RA and rexinoid enhance skeletal myogenesis through differential activation of early developmental genes. Our study demonstrates that activation of RXR causes an increase in the mesodermal Meox1 gene while RA induces the skeletal specific gene Pax3. It will be interesting to uncover other novel genes targeted by rexinoid. Determining the molecular mechanism by which rexinoid exerts its effects to enhance skeletal myogenesis is challenging due in part to the complexity of the developmental systems in which it exerts its effects as well as the intricate relationship of protein complexes and gene regulation. Since ES cells closely recapitulate the properties of the developing embryo, elucidating these molecular pathways will be imperative in the manipulation of stem cell progenitors and aid in the generation of pure populations of skeletal myocytes to use in the treatment of muscle-related diseases.

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Chapter 4

Role and Function of Wnts in the Regulation of Myogenesis: When Wnt Meets Myostatin

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Additional information is available at the end of the chapter

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1. Introduction

Wnt glycolipoproteins are extracellular ligands that can be found in many species, ranging from the sea anemone to human [1]. Wnts are signaling factors regulating several key developmental processes, such as proliferation, differentiation, asymmetric division, patterning and cell fate determination [2,3]. The Wnt family consists of 19 lipid modified secreted glycoproteins that are primarily divided into two main categories based on their role in cytosolic β-catenin stabilization: canonic and non canonic [4,5,6]. During canonical Wnt signaling, binding of Wnt ligands to Frizzled/low-density lipoprotein-related protein (LRP) receptor complexes causes a stabilization of β-catenin, which is normally degraded by axin/glycogen synthase kinase-3 (GSK-3)/adenomatous polyposis coli (APC) complexes. Stabilized β-catenin is then able to translocate to the nucleus and through interactions with the T-cell factor (Tcf)/ lymphoid enhancer factor 1 (LEF-1), modulates the expression of specific genes [7]. These genes, by regulating cell proliferation, differentiation, adhesion, morphogenesis are involved in various essential physiological and physiopathological processes as embryonic and adult development, cellular and tissular homeostasis, and diseases [8,9,10,11,12]. In contrast, the less-characterized non-canonical Wnt pathways are independent of β-catenin and transduce Wnt signals through numerous signaling, including either c-Jun NH2-terminal kinases (JNK)/planar cell polarity or Wnt/calcium pathways [13,14,15,16,17].

During development, Wnts act as morphogens and control the patterning of the embryo by triggering concentration-dependent autocrine and paracrine responses [18,19,20]. Globally, Wnts are involved in the embryonic myogenesis by regulating the activity of myogenic regulatory factors (MRF).

Whereas data are well established concerning the functions of Wnt proteins during embryonic muscle development, the knowledge of the implications of Wnt signaling in

adult muscle regeneration and homeostasis is much less advanced. The ability of skeletal muscle to grow, maintain, and regenerate itself is dependent on a population of satellite cells, a specialized pool of adult myogenic stem cells, that reside in between the muscle basal lamina and the cell membrane of myofibers. Satellite cells generate muscle precursor cells that then proliferate before they either fuse into an existing myofiber to become post-mitotic nuclei or fuse together to form myotubes [21,22,23]. Accumulating data show that among the signaling proteins, members of the Wnt family are strongly implicated in the adult skeletal muscle development, growth and regeneration [24,25,26,27].

The role of Wnt signaling in myoblast differentiation is supported by the fact that myostatin (Mstn), a member of the transforming growth factor-β (TGF-β) superfamily, negatively regulates muscle mass via non-canonical Wnt signaling pathways [28]. Mstn is an important regulator of skeletal muscle development and adult homeostasis. Naturally occurring mutations, as well as experimental knockout of the *Mstn* gene, lead to hypermuscular phenotype [29,30,31]. Later studies have subsequently established that Mstn regulates the size and the number of muscle fibers by inhibiting myoblast proliferation and differentiation [32,33,34,35]. In this context, it has been shown that, among the Wnt genes, Wnt4 was the most responsive to Mstn, both on differentiation of human bone marrow-derived mesenchymal stem cells [36] and on postnatal skeletal muscle growth [28]. Recent studies showed that Wnt4 inhibited Mstn expression and Mstn signaling pathway [37]. Reciprocally, it has been found that the genetic deletion of Mstn renders the satellite cells refractory to the hypertrophic effect of Wnt4, suggesting that the Wnt4-induced decrease of Mstn plays a functional role during hypertrophy [37]. Overall, these results indicate that Wnt factors are modulator of myogenesis and these actions can be mediated through interaction with Mstn.

In this review, we will detail Wnt signaling pathways, their implications during embryonic and adult skeletal myogenesis. A particular attention will be paid to the relation between Wnt factors, particularly Wnt4 and myostatin.

2. Wnt signaling

2.1. Components of the Wnt signaling pathway

2.1.1. Wnts, the ligands

Wnt proteins constitute a large family of cystein-rich secreted glycolipoproteins that control development in organisms ranging from nematode worms to mammals [38]. To date, at least 19 Wnt genes have been identified in mammals; they are similar in size, ranging from 39 kDa to 46 kDa [39,40,41]. All Wnts have a signal sequence followed by a highly conserved distribution of cysteine residues, the spacing of which is highly conserved, suggesting that Wnt protein folding may depend on the formation of multiple intra-molecular disulfide bonds [42,43]. Once secreted, Wnt ligands remain tightly associated with the extracellular matrix, with a strong affinity for heparin sulfate proteoglycans [44,45]. In addition, Wnt ligands are carried on lipoprotein particles to facilitate signaling activities in the intercellular

space [46,47,48]. Although Wnts are secreted proteins, they are highly insoluble and have therefore been difficult to be purified [39,49]. As a consequence, very little is known about the structure and biochemical properties of Wnt proteins. They mediate their signaling activity through the interaction with the seven transmembrane Frizzled (Fzd) receptor and the co-receptor LDL-receptor related protein (LRP) [50,51,52]. The Wnt family members can be divided in two distinct classes depending on their ability to induce transformation of the mouse mammary epithelial cell line C57MG [53]. The highly transforming members include Wnt1, Wnt3, Wnt3a, and Wnt7a. The intermediately transforming or non-transforming members include Wnt2, Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7b, and Wnt11 [54]. These two groups of Wnts signal via intracellular pathways that trigger different developmental outcomes [53]. In mammals, highly transforming Wnts have been associated to the canonical Wnt/β-catenin pathway, whereas non-transforming Wnts have been associated activate to the non-canonical pathway [4,6]. It is noteworthy that this binary classification is becoming obsolete, thus although Wnt4 was originally described as a non-canonical Wnt (non-transforming Wnt), it has also been implicated in the activation or inhibition of the canonical Wnt pathway [55,56,57,58,59,60,61]. Wnt7a has also been implicated in both canonical and non-canonical Wnt signaling depending on the cell and tissue context [62,63]. Non-canonical Wnt signaling pathways are less well defined, but appear to function in a β-catenin independent manner to regulate processes such as convergent extension during vertebrate gastrulation, and planar polarity [64,65,66,67,68].

2.1.2. Frizzled, the receptors

Frizzled genes encode membrane proteins that mediate multiple signal transduction pathways. They have been identified in diverse animals, from sponges to humans. These proteins belong to the family of G protein-coupled receptor proteins which act as receptors for secreted Wnts [51,69,70]. Ten frizzled proteins have been identified in mouse and humans, and all share the following structural similarities: a signal peptide sequence at the amino terminus, a conserved region of 120 amino acids in the extracellular domain containing 10 invariantly spaced cysteines (called the cysteine rich domain CRD), a seven-pass transmembrane region, in which the transmembrane segments are well conserved, and a cytoplasmic domain with little homology among members of the family [71,72,73,74]. The structure of CRD domain has been solved and it appears to be necessary and sufficient for binding to Wnt [74,75]. Fzds are coupled with trimeric G proteins, and the Wnt stimulated pathway is sensitive to inactivation by pertussis toxin. Specificity of the Wnt-Fzd interaction remains largely unknown, particularly in vertebrates, because of the large numbers of Wnts and Fzds [76,77,78].

2.1.3. LRPs, the co-receptors

Low-density receptor-related protein 5 and 6 (LRP-5 and LRP-6), which are highly homologous, are members of the low-density-lipoprotein (LDL) family of receptors [79,80]. LRP functions as a co-receptor for Wnt signaling leading to the activation of the Wnt/β-catenin pathway. LRP-5 and LRP-6 are type I single-span transmembrane proteins

[81,82,83]. LRP-5 and -6, which bind to Axin through their intracellular domain, are key signaling receptors for the β-catenin pathway [84,85]. The binding between LRP5 and LRP6 is directly associated to the stabilization of β-catenin [79].

2.1.4. Secreted modulators of the Wnt pathway

The extracellular antagonists of the Wnt signaling pathway can be divided into two broad classes. The first class, which includes the secreted Frizzled receptor (sFRP), primarily binds to Wnt proteins; the second class comprises the Dickkopf (Dkk) family, which binds to the LRP subunit of the Wnt receptor complex [86,87,88]. Both classes of molecules prevent ligand-receptor interactions by different mechanisms [89]. sFRPs share homology in the N-terminal region with the cysteine-rich domain of Frizzled proteins, but lack the transmembrane domain [90,91]. sFRPs inhibit Wnt signaling by competing with Fzd for Wnt ligands or in a dominant negative fashion by forming a non-signaling complex with Wnt [92]. There are presently five known members of the family in mouse, sFRP1 to sFRP5. sFRPs are composed of a cysteine rich domain (CRD) and a domain that shares weak sequence similarity with the axon guidance protein netrin (NTR) in the C-terminus [93,94]. The CRD shares 30-50% sequence similarity with those of Fzd proteins and includes 10 conserved cysteine residues.

The four members of the Dickkopf (Dkk) family (Dkk-1 to Dkk-4), inhibit Wnt signaling by binding to the LRP-5 and LRP-6 components of the receptor complex [87,95]. Dkks contain two characteristic cysteine-rich domains (Cys-1 and Cys-2) separated by a linker region of variable length. Cys-2, in particular, is highly conserved among all 69 members of the family and contains 10 conserved cysteine residues [88,89].

2.1.5. Dishevelled

Dishevelled (*Dvl*) is a phosphoprotein essential for the transduction of the Wnt signaling pathway. The *Dvl* family comprises three *Dvl* proteins (Dvl-1, Dvl-2, and Dvl-3). The structure of *Dvl* family members consist of three highly conserved domains: an amino-terminal DIX domain, a central PDZ domain, and a carboxy DEP domain [96]. *Dvl* act as a key transducer of the Wnt signal and act at the plasma membrane or in the cytoplasm. *Dvl* is differentially targeted to participate in either Wnt/β-catenin or PCP signaling [6,7,97]. Activation of a specific pathway through *Dvl* depends on its subcellular localization and activation of modulator downstream. In Wnt/β-catenin pathway, once Wnt binds to the Fzd transmembrane receptor and the co-receptor LRP5/6, *Dvl* can interact with the Fzd/LRP complex. *Dvl* is phosphorylated by casein kinase Iε to form a complex with Frat1 and inhibits GSK3β activity, leading to stabilization of cytoplasmic β-catenin.

The Planar cell polarity pathway makes use of *Dvl* to modify the actin cytoskeleton [98,99]. At the level of *Dvl*, an independent and parallel pathway leads to the activation of the small GTPases Rho [66]. Rho signaling occurs through the molecule Dishevelled associated activator of morphogenesis 1 (DAAM1) [66]. The Rho pathway leads to the activation of Rho associated kinase, which mediates cytoskeletal reorganization [6,99].

2.2. The canonical or Wnt/β-catenin pathway

Wnt family glycoproteins are primarily divided into two main categories based on their role in cytosolic β-catenin stabilization: canonic and non-canonical [4,5,6,100] (Figure 1). Signaling through the Wnt/β-catenin pathway also called canonical pathway, modify the stabilization of β-catenin. In the absence of Wnt binding to Frizzled receptors, β-catenin is targeted for degradation by a multi-protein destruction complex composed of the tumor suppressor protein APC (*adenomatous polyposis coli* gene product), axin, and glycogen synthase kinase 3β (GSK3β) [101,102]. This process is triggered by phosphorylation of β-catenin by the serine/threonine kinases, GSK3β and Casein Kinase [103]. In the complex, the interaction between these kinases and β-catenin is made easier by the scaffolding proteins Axin and APC [104,105,106,107]. Phosphorylated β-catenin is recognized by β-transducin repeat containing protein (β-TrCP), targeted for ubiquitination, and degraded by the 26S proteasome [108,109]. Binding of Wnts to its receptors Frizzled and its co-receptor LRP5/6, inhibits the kinase activity of the destruction complex, and leads to the stabilization of non-phosphorylated β-catenin [110,111]. This mechanism involves either the recruitment of Axin to the plasma membrane after the phosphorylation of LRP5/6, or the action of an axin-binding molecule, Dishevelled (*Dvl*) [96,112]. Kinase inhibition leads to the accumulation of free cytosolic β-catenin. The elevated cytosolic β-catenin can translocate to the nucleus, where it interacts with the N-terminus of the DNA-binding proteins of the T-cell factor/Lymphoid enhanced factor (Tcf/Lef) family [113,114,115]. This transient interaction with β-catenin converts Tcf/Lef factors into transcriptional activators. In the absence of Wnt, Tcf/Lef proteins repress target genes through a direct association with co-repressors such as Groucho [102,116,117].

2.3. Non-canonical signaling pathways

In contrast, non-canonical Wnts transduce their signal independently of β-catenin. The non-canonical Wnt signaling pathway has been found to be associated with gastrulation movements, heart induction, dorsoventral patterning, tissue separation and neuronal migration [6]. Unlike the canonical Wnt signaling pathway, the non-canonical Wnt signaling pathway is quite diverse. Thus, it has been reported that non-canonical Wnts can activate calcium flux, G proteins, Rho GTPases, or c-Jun N-terminal kinase (JNK) [6,66,82].

The response to a given stimulus depends not only on which Wnt is present, but also on which cognate receptor is expressed on the cell [118], It is likely that one Wnt protein can signal more than one type of response in a cell if multiple types of receptors are present [58]. Adding still more layers of complexity, cofactors, secreted antagonists and co-receptors of Wnt signalling are likely to affect both canonical and non-canonical actions [89,119].

2.3.1. Calcium pathway

Activation of this signaling pathway involves Wnt binding to a Frizzled receptor, subsequent release of intracellular calcium associated with the activation of various enzymes such as $Ca2^{+}$/calmodulin-dependent (CamKII) protein kinase and Protein Kinase C (PKC) [6,120,121,122] (Figure 1). Here, frizzled receptors act through G-protein and activate phospholipase C (PLC)

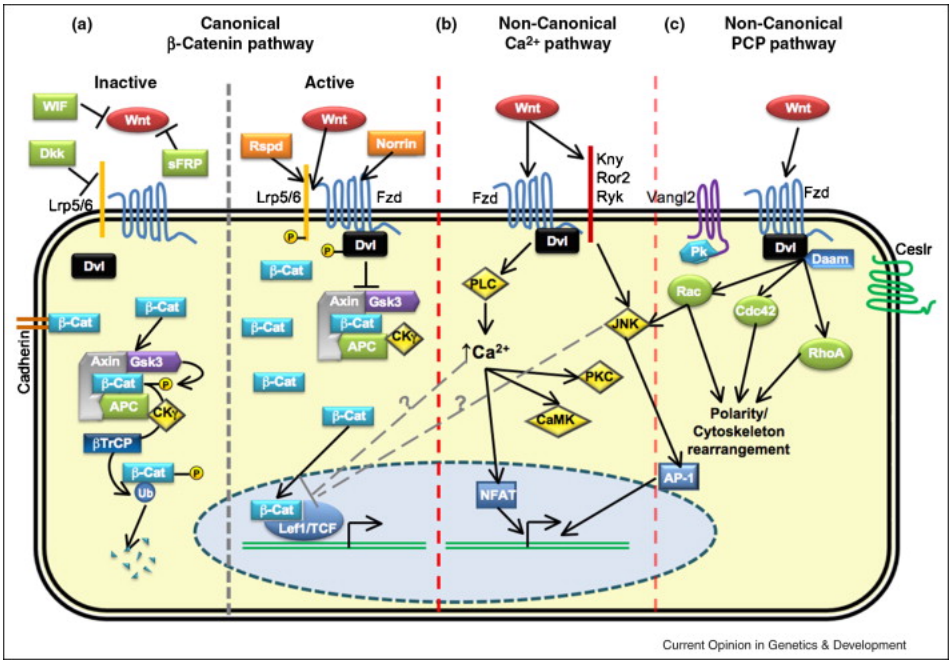

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Figure 1. Three Wnt-dependent pathways have been proposed: (a) canonical Wnt/β-catenin pathway and (b and c) non-canonical Wnt/PCP and Wnt/Ca2+ pathways. Canonical and non-canonical pathways possess clear different signaling events; however, the distinction between Wnt/PCP and Wnt/Ca2+ pathways is less obvious and common events occur on those pathways. (Dashed lines illustrate the idea that no clear boundaries exist between the different Wnt pathways.) (a) Canonical Wnt/β-catenin pathway. In cells, β-catenin is normally associated with adherens junctions and can also be free in cytoplasm. In cells non-stimulated by Wnt ligands (which can additionally be inhibited by WIF, sFRPs and Dkk protein family members) cytosolic β-catenin is targeted to proteolytic degradation through phosphorylation by the APC–Axin–GSK3 β–CK1γ complex and further ubiquitination through action of βTrCP-dependent E3 ubiquitin ligase complex. On stimulation by Wnt ligands though binding to Fzd receptors and its co-receptors Lrp5/6, Fzd recruits Dvl. Dvl will inhibit APC–Axin–GSK3β–CK1γ complex formation by the recruitment and inhibition of GSK3β, CK1γ and Axin to the cytoplasmic membrane. Consequently, β-catenin can accumulate in the cytoplasm and enter the nucleus, activating transcription of target genes through association with Lef1/TCF transcription factor family. (b) Non-canonical Wnt/Ca2+ pathway. Interaction of Wnt ligands with Fzd receptors can lead to an increase in intracellular calcium level, through possibly the activation of PLC. Intracellular calcium will subsequently activate CAMKII and PKC in cells, as well as the transcription factor NFAT. This pathway is particularly important for convergent-extension movements during gastrulation. Additionally, Fzd receptors in association with Kny, Ror2 or Ryk receptors can also activate JNK promoting expression of specific genes through activation of AP-1. (c) Non-canonical Wnt/PCP pathway. This pathway is characterized by an asymmetric distribution of Fzd, CELSR, Pk and VANGL2, resulting in the polarization of the cell. Also, Wnt-signaling activates Rho GTPases Cdc42, RhoA and Rac1 leading to cytoskeleton rearrangement, with the participation of Daam1. Rac1 can also activate JNK, activation-specific gene transcription through modulation of AP-1 protein complex.

and phosphodiesterase (PDE), which lead to increased concentrations of free intracellular calcium and to decreased cyclic guanosine monophosphate (cGMP) [121,123,124]. Elevated free intracellular calcium can activate the phosphatases calcineurin, leading to dephosphorylation and activation of the transcription factor NFAT [14].

2.3.2. Planar cell polarity pathway

The planar cell polarity (PCP) pathway regulates the orientation and migration of polarized cells during development and defects underlie severe abnormalities [66,67,125]. Although, the regulation of this pathway appears to be incredibly complex, in vertebrates, it signals mainly through the JNK pathway. Upon binding of Wnt to its receptor Frizzled, there is activation of Disheveled, JNK and Rho family GTPases, which direct asymmetric cytoskeletal organization and coordinated polarization of cells within the plane of epithelial sheets [68,126,127] (Figure 1).

3. Implication of Wnt signaling in skeletal myogenesis

3.1. Embryonic skeletal myogenesis

Like any process of tissue development, skeletal muscle development is under the control of two regulation pathways: an intrinsic pathway implicating transcription factors (Pax3, Pax7, Myf5, MyoD1, Mrf4, Myogenin, Six proteins, SRF….) and an extrinsic pathway implicating external factors as Wnt, sFRP, Myostatin, Sonic Hedgehog (*Shh*), BMP, Notch, FGF, HGF/Met, insulin, IGF1, retinoic acid….

3.1.1. Intrinsic pathway

Muscles of the trunk and limbs of vertebrate embryos are derived from somites. These segmental masses correspond to a series of transient repeated epithelial structures that derive from the paraxial mesoderm and lie on either side of the neural tube [128]. Somites eventually differentiate into five major cell types: cartilage, bone, and tendons of the trunk, skeletal muscles of the body and the dermis of the back [128,129,130,131]. This process is regulated by sonic hedgehog (*Shh*) and Wnt signals that are secreted by tissues surrounding the somites [132,133]. This process leads to patterning of the epithelial structures into distinct compartments that give rise to diverse cell lineages [129,131,133,134]. The compartments formed are (i) the mesenchymal sclerotome which contributes to cartilage and bone of the vertebral column and ribs and (ii) the dorsally located epithelial dermomyotome where myogenic precursors are localized and ultimately give rise to epaxial (back) and hypaxial (muscles of the ventral body wall, limbs, diaphragm and tongue) muscles [134,135]. All cells of the dermomyotome, including the epaxial and hypaxial zones, are initially positive for the Pax3 transcription factor, whereas Pax3/Pax7 double positive cells are only located in the central domain [136]. Myogenesis is initiated by the translocation of myogenic progenitors that migrate as Pax3 positive cells from the extremities of the dermomyotome to the myotomal layer below or as Pax3/7 double positive

cells from the central dermomyotome to the myotome [128,133,137,138,139,140,141]. These cells enter the myogenic program after activating of the myogenic regulatory factors (MRFs): Myf5 and Mrf4 [2,142]. Double mutant Pax3/Pax7 mouse embryos suffer from a major skeletal muscle deficit where only the early myotome is formed, but the cells in these structures do not activate Myf5 or MyoD and consequently fail to enter the myogenic program consequently, they die or migrate towards a non myogenic fate [143,144]. Pax3 and Myf5/Mrf4 control the activation of MyoD, another MRF family member and MyoD positive myogenic precursors differentiate into myofibers or remain as a proliferating population called satellite cell population [145,146].

3.1.2. Extrinsic pathway

Numerous environmental paracrine factors are associated to the regulation of skeletal myogenesis, thus, for example, Notch signaling plays a role in cell fate determination in the lateral dermomyotome [147], in the amplification of Pax3+/Pax7+ progenitors cells and in the myoblastic proliferation [148].

Overall, Wnt protein family has been determined along with *Shh* to be the main molecules required to activate myogenesis [149,150,151,152] (Figure 2).

In response to *Shh* and Wnt signals from the notochord and neural tube, somites delaminate to form the sclerotome and dermomyotome. The notochord and the floor plate of the neural tube secrete ventralizing signals, including *Shh* whereas the surface ectoderm and the dorsal neural tube secrete Wnt proteins [153]. These two morphogens will induce the induction of MRFs leading to the delamination, specification and myoblast differentiation.

In a remarkable study realized from chicken cultures of presomitic mesoderm cells, A.E Munsterber et al. have shown that Wnts could mimic the inducer effect of the dorsal neural tube and that *Shh* could mimic the effect of the floor plate and the notochord [25,154]. These results indicated that Wnt1, Wnt3, Wnt4 and *Shh* could replace the inducer role of the dorsal neural tube and the floor plate/Notochord structures. Ectopically implanted Wnt1, Wnt3a and Wnt4 expressing cells in chicken alter the process of somite compartmentalization *in vivo*, resulting in an enhanced recruitment of somitic cells into the myogenic lineage [155]. In the same study, authors reported no difference on somite development between neural tube (expressing Wnt1 and 3a) and surface ectoderm (expressing Wnt4), suggesting that surface ectoderm promote myogenesis by Wnt4 secretion [155].

In mouse, similar experiments indicated that the expression of Myf5 is induced by a signal emanating from neural tube and that the expression of MyoD is depending of a signal coming from surface ectoderm [156]. Wnt7a secreted by the surface ectoderm and Wnt1/Wnt3a secreted by dorsal neural tube regulate MyoD and Myf5 expression, respectively whereas Wnt4 and Wnt5a regulate both MyoD and Myf5 expression [132]. Furthermore, it has been shown that Wnt1, Wnt3a and Wnt4 are expressed in the dorsal half of the neural tube when epaxial myogenesis is initiated [153]. Regulation of hypaxial muscle specification is less understood but Tajbakhsh et al, 1998 have shown that Wnt7a and to a

lesser extent Wnt4 and Wnt5a can activate myogenesis in mouse paraxial mesoderm explants [132]. Conversely, sFRPs, inhibitor of Wnt signaling, markedly reduce myogenesis by antagonizing Wnts [157,158].

Use of somites from transgenic mice expressing lacZ reporter gene under the control of Myf5 epaxial promoter in presence of a constitutive form of β-catenin or in presence of the neural tube lead to a strong activation of the reporter, indicating that the Wnts secreted by the neural tube act mainly through canonical pathway [159]. In the surface ectoderm, signaling pathway initiated by Wnt7a differs from that by Wnt1. Wnt7a binds to Frz7 and signals through Protein kinase C, a β-catenin independent pathway [122,149]. Recently, the Wnt PCP pathway has been implicated in regulating the orientation of myocyte growth in the developing myotome [160]. As reported during osteogenic differentiation or during recruitment, maintenance, and differentiation of human bone marrow mesenchymal stem cells a cross-talk between canonical and non-canonical Wnt signaling exists and may lead to functional antagonism [161,162].

In chick embryos, Nohno's group analyzed the myogenic effects of Wnt4 overexpression in the limb bud in order to evaluate the significance of this factor in skeletal muscle formation during embryogenesis. They found that Wnt4 treatment induced an increase in the muscle mass, particularly in fast-type muscle size. Furthermore, ectopic Wnt4 induces muscle satellite markers Pax7 and MyoD expression [60].

As extrinsic factors, myostatin (Mstn), a member of the TGF-β superfamily, has been proposed as a regulator of embryonic myogenesis [30,32,34,163,164,165,166]. Myostatin, also called GDF8 (growth differentiation factor 8), is a secreted growth factor that belongs to the transforming growth factor-β (TGF-β) superfamily of growth and differentiation factors [30]. In mice, myostatin is predominantly expressed in skeletal muscle tissues from the period of embryogenesis to adulthood suggesting a role for this factor in the control of muscle development and function [30,166]. The role of myostatin in muscle comes from the phenotype of myostatin-deficient animals. Myostatin was first found to regulate muscle mass in mice from which the gene encoding myostatin has been knocked-out. The resulting "mighty mice" displayed muscle overgrowth due to both hyperplasia (increased number of muscle fibers) and hypertrophy (increased size of individual muscle fibers). These effects on muscle mass are persistent throughout the life of the animals. The phenotype of these mice suggested that myostatin functions as a negative regulator of muscle growth. Interestingly, the function of myostatin appears to have been conserved across diverse species. Natural mutations in the myostatin gene have been identified in double-muscled animals such as the Belgian blue cattle [29,167,168]. The recent identification of a hypermuscular child with a loss-of-function mutation in the myostatin gene suggests that the function of myostatin is similarly conserved in humans [169]. In support of this, myostatin sequence has been highly conserved through evolution, among species ranging from zebrafish to humans [30]. At early embryonic stages, myostatin expression is restricted to the myotome compartment of the developing somites, and myostatin has been proposed to play an essential role in skeletal muscle growth and development [30,165].

It is of note that many others autocrine or paracrine factors, such as Notch, Tgf-β1, Bmp, Noggin, mi-RNA…, play a subtle but crucial role in orchestrating the regulation of embryonic myogenesis [170,171,172,173,174,175,176]. Undoubtedly, the complete decoding of this intricate network will take several years to be complete.

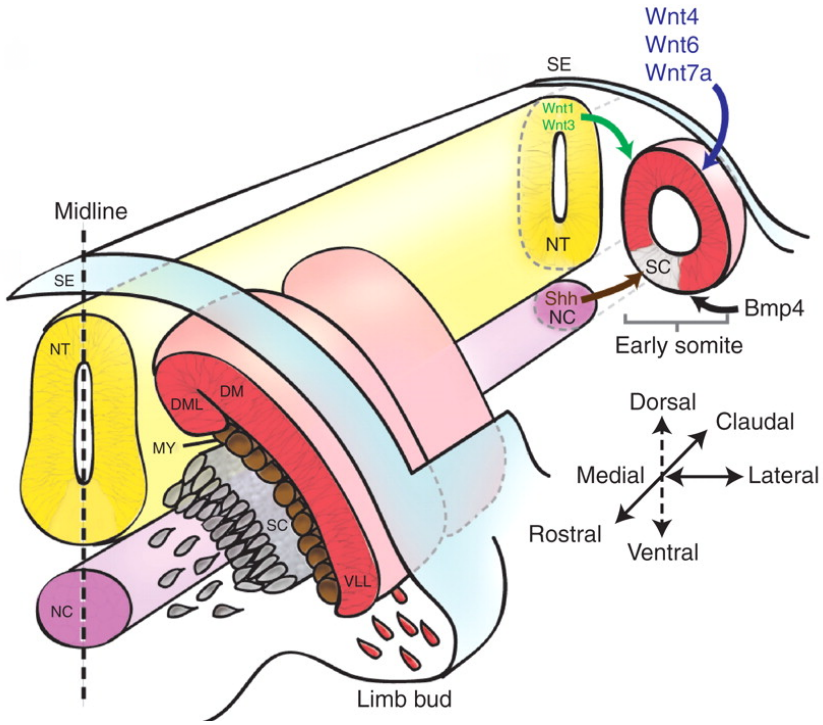

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Figure 2. Embryonic myogenesis. Illustration of the morphogen gradients along the rostral–caudal axis of the embryo. (C) Schematic of transverse sections through the embryo at early (i) and late (ii) stages of somitogenesis. (Ci) Morphogens secreted from various domains in the embryo specify the early somite to form the sclerotome (SC) and dermomyotome (DM). Wnts secreted from the dorsal neural tube (NT) and surface ectoderm (SE) along with bone morphogenetic protein (BMP) from the lateral plate mesoderm maintain the undifferentiated state of the somite, whereas Sonic hedgehog (Shh) signals from the neural tube floor plate and notochord (NC) induce the formation of the sclerotome. (Cii) As the sclerotome segregates, muscle progenitor cells (MPCs) from the dorsomedial (DML) and ventrolateral (VLL) lips of the dermomyotome mature give rise to the myotome (MY). At the level of the limb bud, Pax3-dependent migrating MPCs delaminate from the ventrolateral lips to later give rise to limb muscles.

3.2. Postnatal myogenesis

3.2.1. Satellite cells

The main role of satellite cells during the early postnatal period is to provide myonuclei for skeletal muscle growth. They contribute to the postnatal growth of syncital muscle cells,

which in adults contain approximately 6–8 times more nuclei than in neonates [177]. In adult muscle, satellite cells are mitotically quiescent and do not express myogenic regulatory factors (MRFs) [178,179]. Their role changes to one of providing myonuclei for homeostasis and hypertrophy or in response to the more sporadic demands for myofiber repair and regeneration [180]. In the latter context, satellite cell activation is dramatically enhanced; they reenter the cell cycle and proliferate [181,182], at this stage they are often referred to as either myogenic precursor cells or myoblasts [182,183]. Some resulting myoblasts differentiate and fuse to form replacement myofibers, whereas some remain as continual dividing cells to replenish the pool of satellite cells in anticipation of future rounds of regeneration [182,184].

Pax7 is expressed in quiescent as well as activated satellite cells, and it is down regulated when satellite cells commit to muscle differentiation [177,185,186]. Compared to embryonic satellite cells, quiescent adult satellite cells do not require Pax7 for self-renewal and regeneration [187]. The progression of activated satellite cells toward myogenic differentiation is mainly controlled by Myf5 and MyoD [188] and is followed by fusion into regenerating fibers. Satellite cells undergo different fates, giving rise to a few Pax7+MyoD– cells, which return to quiescence (satellite stem cell population), and many Pax7+MyoD+ cells, which differentiate [189]. These asymmetric divisions are mainly regulated by Notch signaling [190,191,192]. In addition, satellite stem cells which represent approximately 10% of Pax7+ mouse satellite can undergo planar symmetric cell divisions to drive expansion of their population [190]. By a subtractive hybridization approach, Legrand et al. found that Wnt7a is expressed during muscle regeneration and acts through Fzd7 receptor to induce symmetric satellite stem cell expansion and thus enhance muscle regeneration. Wnt7a signaling though Frz7 receptor requires association with Vangl2, a membrane protein involved in the regulation of PCP suggesting that Wnt7a utilizes this non-canonical pathway to control the orientation of satellite cell division [62]. They suggest that Wnt7a regulates the homeostatic maintenance of the satellite stem cell pool by modulating the increase in satellite stem cell expansion during regenerative myogenesis and that basal levels of PCP signaling are insufficient to maintain the satellite cell pool at normal levels [62].

Aging is accompanied by a decline in muscle mass and strength, a phenomenon referred as to sarcopenia [193]. It is clear that fitness is greater at any age in individuals who exercise regularly versus those who do not and that sarcopenia is reduced in physically active elderly people. A decline in the number of satellite cells, their proliferative capacities, or both, may contribute to sarcopenia. One of the mechanisms responsible for the reduced regenerative potential of old muscle seems to be the decline in Notch signaling [194]. Aging of skeletal muscle is characterized by an increase in fibrous connective tissue and adipose tissue and by an impairment of muscle regenerative potential [195,196]. This decrease of aged-muscle regeneration can be enhanced by direct activation of the Notch pathway [194] or by exposure to a youthful systemic environment [197]. Very interestingly, these age-related effects are associated with increased canonical Wnt signaling in the satellite cell population, possibly resulting from increased amounts of Wnt or Wnt-like molecules in the serum of aged animals [24]. This generalized role of Wnt signaling in promoting an aging phenotype is consistent with the findings of Liu et al. [198].

3.2.2. Non satellite cells

Interestingly, a variety of non-satellite cell, located outside the basal lamina can also participate in skeletal muscle regeneration in the adult. These include pericytes, endothelial cells, mesoangioblasts [177,199,200,201,202], and interstitial cells (PICs) [203], as well as other cell types that are not fully characterized [26,204,205]. Polesskaya et al. found that a side population of cells harboring surface markers CD45 and Sca1 (CD45+:Sca1+) isolated from regenerating muscle readily underwent myogenic differentiation *in vitro* [26]. As quiescent satellite cells, activated satellite cells and satellite cell derived myoblasts do not express CD45 and satellite cell lineage do not express Sca1, CD45+:Sca1+ cells constitute a cellular pool distinct of muscle satellite cells. The fact that (i) activation of the canonical Wnt pathway by LiCl in isolated CD45+:Sca1+ cells was sufficient to induce muscle specification, (ii) myogenic commitment of CD45+:Sca1+ cells were induced by co-culture with cells ectopically expressing Wnt proteins and, (iii) the number of CD45+:Sca1+ cells was decreased in injured muscle treated with sFRPs, demonstrated clearly a functional requirement for canonical Wnt in the myogenic specification of CD45+:Sca1+ cells. These exciting results establish that myogenic progenitors derived from non-satellite cell can have a physiological role in muscle regeneration.

3.2.3. Wnt-related regulation of myoblast proliferation and differentiation

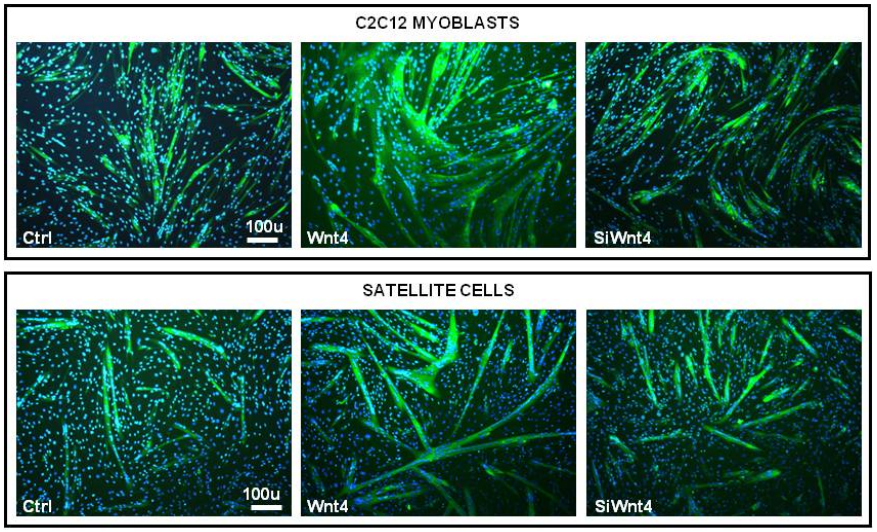
In addition to the crucial role of Wnt proteins in satellite cells fate and maintenance, they play an essential role in the control of proliferation and differentiation in various cellular types.

In order to better characterize the role of Wnt proteins in the control of muscle cell differentiation, Bernardi et al. established Wnt expression pattern on C2C12 myoblasts and satellite cells [37]. They found that, among the 19 existing Wnts, only the expression of Wnt4 was strongly activated from the early steps of differentiation. Interestingly, by modulating Wnt4 expression level with overexpression or silencing approaches, they reported a strong differentiation promoting activity of this factor (Figure 3). The relative increase in myotube size being comparable 48, 72, and 96 h after switching to differentiation medium indicates that Wnt4 signaling controls myotube size at the early steps of differentiation during myotube formation. The lack of Wnt4-mediated hypertrophic effect observed when cells were transfected the day of differentiation or later indicates that this factor has to be expressed during the proliferative stage to be effective.

Fusion consists of two distinct phases: myoblast/myoblast fusion to form nascent myotubes and subsequent myoblast/myotubes fusion, resulting in a rapid accretion of size. The efficiency of the first phase can be evaluated by measuring the fusion index, which represents the proportion of the total cell population that has fused. After being switched to differentiation medium, Wnt4 overexpression in C2C12 and satellite cells gave a 62% and a 67% fusion index increase, respectively, suggesting that Wnt4 signaling control nascent myotube formation. Recently, Tanaka et al. confirms that Wnt4 overexpression increased troponin-T positive cells in proliferation medium and enhanced myotube formation of differentiated C2C12 cells [206].

Takata et al. reported that Wnt4 exhibited a low but significant myogenic activity with an increase of proliferation rate in myoblast cells [60]. Conversely, Otto and co-workers found, using co-culture of Wnt4-producing NIH-3T3 cell line on single muscle fiber culture, a 24% reduction of satellite-cell proliferation [207]. These conflicting results could be attributed either to the different specificities of the cellular models used or to the fact that effective concentration and physiological activity of ectopicWnt4 can be eminently variable, depending on the nature and activity of produced Wnt4. Taken together, these data are in agreement with a modest effect of Wnt4 on proliferation, suggesting a role of this factor in enhancing cell fusion rather than controlling the number of nuclei available for fusion.

Knockdown of Wnt4 expression in C2C12 and satellite cells inhibits their differentiation and further confirms that Wnt4 acts as an effector of myogenesis (Figure 3). However, addition of siWnt4 after switching to differentiation medium or 24 h before did not decrease significantly the differentiation rate. This confirms that Wnt4 acts as a positive regulator of myogenic differentiation only if it is expressed in the proliferative phase.



From Bernardi et al. Am J Physiol Cell Physiol-2011

Figure 3. Effects of Wnt4 overexpression and Wnt4 silencing on C2C12 myoblasts and satellite cells differentiation. Stably transfected polyclonal C2C12 myoblasts with empty (Ctrl) or Wnt4-containing (Wnt4) expression vector were grown 2 days in proliferation medium followed by 4 days in differentiation medium. C2C12 myoblasts and satellite cells transfected with siRNA specific to Wnt4 (siWnt4) and negative control siLuc were grown 2 days in proliferation medium followed by 4 days in differentiation medium. Fluorescent images of C2C12 myoblasts were obtained by immunostaining with a monoclonal anti-myosin antibody. Nuclei were labeled by DAPI staining.

Myoblastic differentiation involves two major steps, their reversible withdrawal from cell cycle and the subsequent expression and activation of myogenic factors. Myf5 and MyoD are expressed in early myogenesis. As cells progress toward a differentiated phenotype,

myogenin and MRF4 are induced and cooperatively establish the irreversible commitment to terminal differentiation. Myf5 is a factor reported to be high in the Go phase, where cells were arrested to differentiate [208]. Borello et al. have previously showed a direct regulation of Myf5 expression by canonical Wnt signaling during somitogenesis [2]. Bernardi et al. found that the expression of Myf5 was activated by Wnt4 overexpression and strongly inhibited by Wnt4 silencing during C2C12 myoblast and satellite cells differentiation, suggesting that myogenic activity of Wnt4 is related to an increase of Myf5 expression.

These authors by measuring Tcf/LEF gene canonical reporter activity and axin2 expression (a protein induced by canonical Wnt pathway), in the presence or absence of canonical Wnt pathway stimulators (LiCl or BIO), reported canonical Wnt pathway activation by Wnt4. Although this factor was originally described as a non-canonical Wnt, it has also been implicated in the activation or inhibition of the canonical Wnt pathway [55,56,57,58,59,60,61]. Numerous reports showed that Wnt-related myogenic activity is mediated mainly through a stimulation of canonical signaling. Thus, Takata et al. found that myogenic activity of Wnt4 is linked to an increase of β-catenin signaling [60]. Likewise, Armstrong et al. have reported that the expression of β-catenin is necessary for physiological growth of skeletal muscle and that Wnt signaling pathway induces β-catenin activation of growth-control genes during overload induced skeletal muscle hypertrophy [209,210]. By using C2C12 and satellite cells, Han et al. reported recently that activation of canonical pathway by R-spondin2, promote myogenic differentiation and hypertrophic myofiber formation [211]. When LiCl was added to the culture medium, the area of myotubes and index fusion were increased in both C2C12 and satellite cells, reaching values similar to those observed after Wnt4-induced hypertrophy [37]. On the other hand, the fact that LiCl exerts a higher Tcf/LEF activation than Wnt4 treatment, whereas the extent of differentiation is the same suggests that this process is dependent on other signaling pathways. In this respect, work of Otto et al. (61) on isolated muscle fibers showed that Wnt4 inhibited satellite cell proliferation but that canonical Wnts such as Wnt1, Wnt3a, and Wnt5a induced a greater degree of proliferation than control cells, confirming the implication of other pathways in Wnt4 signaling [207]. The stronger activity in terms of Tcf/LEF activation of LiCl compared with Wnt4 (+100% on C2C12 myoblasts, +250% on satellite cells) treatments could explain the discrepancy between proliferative activities of Wnt4 and LiCl. Indeed, as described above, Wnt4 shows a weak effect on mitogenicity, whereas 10 mM LiCl has a strong anti-proliferative activity (-15% on satellite cells, -48% on satellite cells), suggesting that low levels of Tcf/LEF activation regulate myogenic differentiation, whereas higher levels regulate preferentially the proliferation. A study reported by Anakwe et al. is also in agreement with a relation between the level of Tcf/LEF activity and the type of cellular response elicited [209]. They reported an increase in the number of terminally differentiated cells in Wnt4 transfected myogenic cells, whereas the overexpression of canonical Wnt3a decreases the number of terminally differentiated myogenic cells. Making more complex the Wnt4-related signaling in muscle, Tanaka et al. showed very recently that Wnt4 promotes myogenesis and suppresses canonical Wnt signaling, culture conditions and C2C12 myoblasts characteristics may be at the origin of

this discrepancy and suggest that data emanating from established cell lines should be considered with care [206]. Moreover, the analysis of cellular response during myoblast differentiation can be made more complex by the implication of other signaling pathways like the non-canonical Wnt/calcium signaling.

Recently, an elegant work from Rudnicki's group showed that Wnt signaling is also implicated in the control of muscular hypertrophy [27]. Skeletal muscle mass depends upon a dynamic balance between anabolic and catabolic processes. Muscle hypertrophy is characterized by an increase of the diameter of muscle fibers and increased protein synthesis, mainly by activation of the IGF1/Akt/mTORC1 pathway. They observed that Wnt7a binding to Fzd7 receptor directly activates the Akt/mTOR growth pathway, thereby inducing myofiber hypertrophy. Besides, they found that anabolic activity of Wnt7a is related to the activation of a new non-canonical pathway implicating stimulatory G protein, Gαs.

3.2.4. Relation between Wnt4 and Myostatin

Myostatin acts through the receptor-associated proteins Smad2 and Smad3 [33,212,213]. Phosphorylated Smad2 and Smad3 form heterodimeric complex with the common mediator Smad4. These activated Smad proteins function as the key intracellular mediators of signaling for myostatin as they translocate into the nucleus, and activate the transcription of the target genes through interaction with DNA and other nuclear factors [214,215]. Takata et al. reported that Wnt4 had no effect on Smad2 phosphorylation, but it antagonized Smad2 phosphorylation induced by Mstn in differentiated C2C12 myoblasts [60]. They suggest that Wnt4 exert its myogenic effect by acting against Mstn. By using CAGA reporter assay, Bernardi et al. confirmed the inhibition of Mstn-activated Smad signaling pathway by Wnt4 [37]. Contrary to differentiated myoblasts, Wnt4 decreased the CAGA reporter activity in proliferating myoblasts below the basal level meaning that Wnt4 has a stronger anti-Mstn activity during proliferation than during differentiation. Furthermore, the fact that the addition of Mstn did not reverse the Wnt4-induced CAGA reporter inhibition in myoblasts and satellite cells suggests that Mstn acts upstream of Wnt4 or that Wnt4 regulates the Mstn/Smad pathway independently of Mstn. The inhibition of the Mstn pathway by Wnt4 can be associated with the inhibition of Mstn expression and/or inhibition of the Mstn/Smad transduction pathway. By using sqRT-PCR and promoter reporter experiments, authors showed that Wnt4 acted as an inhibitor of Mstn expression. Moreover, study with LiCl, strongly suggests that Wnt4 regulates negatively Mstn expression through activation of the canonical β-catenin pathway. However, while Wnt4 down-regulates Mstn expression in proliferative and differentiated cells, the activation of canonical signaling by LiCl inhibits Mstn expression only when cells are in proliferative or early differentiated states. Thus, in function of the differentiation state, various signaling pathways can be implicated in the inhibition of Mstn expression by Wnt4 in myogenic cells. A biphasic mode of action of Wnt4 can be proposed: in proliferative and early differentiated stages, Wnt4 acts by inducing canonical signaling, and later in the differentiation, canonical pathways is relaxed and another pathway maintains Mstn at low level. However, the fact that Wnt4 displayed the strongest CAGA reporter inhibiting activity in proliferative myoblasts, whereas Wnt4 had

the strongest Mstn expression inhibiting activity in differentiated myoblasts, implies that Wnt4 regulates both Mstn expression and Mstn/Smad signaling pathway. In silico analysis in the Mstn promoter revealed the presence of three putative Tcf/LEF binding sites within a 2-kb sequence upstream of the *Mstn* gene at position -1889, -1028, and -743 confirming the observed regulation of the Mstn expression by Wnt/β-catenin pathway. Analyze of reciprocal regulation of the both signaling pathways showed that whereas Mstn inhibits the accumulation of nuclear β-catenin in differentiated C2C12 [36], Mstn was not able to inhibit Tcf/LEF activity induced by Wnt4 overexpression in proliferative and differentiated C2C12 myoblasts [37]. Thus, contrary to adipogenic differentiation of human bone marrow-derived mesenchymal stem cells in which there is a cross-communication of the Mstn signal with Wnt/β-catenin pathway, Wnt4/β-catenin signaling pathway is not affected by Mstn.

Mstn has been described as an inhibitor of myotube formation in C2C12 and satellite cells [32,33,34,35]. The reversion of Wnt4 activity by recombinant Mstn and the absence of hypertrophic activity of this factor in Mstn-/- mice demonstrated clearly that Wnt4 requires the presence of Mstn to elicit its differentiation promoting activity [37]. Thus the negative regulation of Mstn by Wnt4 is a crucial step for the myotube formation and hypertrophy. The fact that the addition of Mstn reverses the hypertrophic activity of Wnt4 but does not reverse the Wnt4-induced CAGA reporter inhibition implies that *1*) Wnt4 acts upstream of Mstn and that *2*) Wnt4 inhibits CAGA reporter by another pathway than Mstn/Smad signaling. Furthermore, the demonstration that Wnt4 can dominantly overcome the expression and activity of atrophic Mstn opens possibilities of the manipulation of Wnt4 or β-catenin levels as a likely target for therapeutic design.

4. Conclusion

Development, growth and maintenance of muscle mass are critical for long-term health and quality of life. In this regard, activation of satellite cells and regulation of anabolic/catabolic muscular pathways play key roles. Production of new myofibrils and degradation of existing proteins is a delicate equilibrium, which, depending on the condition, can promote muscle growth or loss. In this matter, skeletal muscle has the capacity to continuously regulate its size in response to a variety of external cues and serves as the most significant repository for protein in the body of healthy adult humans (~50% total protein content). To achieve a correct development and to maintain homeostasis, the biological response of the skeletal muscle implies a multitude of regulatory signaling pathways that orchestrates myogenesis in embryonic development and enables adult muscle repair. There is much interest in understanding the cellular and molecular mechanisms underlying skeletal muscle homeostasis and regeneration in different contexts because such knowledge might help in the development of cell therapies for diseases characterized by skeletal muscle degeneration. As described in this review, it is clear that Wnt play multiple and essential actions both in developmental and postnatal muscles. Depending not only of the Wnt signaling pathways implicated but also of the other interacting signaling pathways, Wnt proteins can act to promote or inhibit myogenesis. Further study will be necessary to elucidate the complex network that regulates embryonic and adult myogenesis.

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Molecular Biology

Chapter 5

Role of Heat Shock Proteins in Skeletal Muscle

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Additional information is available at the end of the chapter

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1. Introduction

The ability of the human organism to respond with rapid and appropriate modification during physiological challenges is an essential feature for its own survival. These modifications are inseparable from a satisfactory adjustment of the physiological processes of the whole body, where physiological systems tend to maintain relatively constant composition of the internal environment (milieu intérieur), despite the constant challenges that the body is submitted daily to, which is known as homeostatic control [1, 2].

The homeostatic regulation of any physiological variable depends on cooperative mechanisms which are activated simultaneously or in succession. Thus, intense or critical challenges to life require numerous and complex mechanisms to restore or to maintain the homeostasis. In this way, challenges generate a stress situation to the body and, depending on the severity, can cause harmful effects. Moreover, moderate challenges, which are also caused by stress, results in profitable physiological adjustments [3]. In this context, stress response may be considered a nonspecific response of the body to any demand. A stressor is an agent that produces stress at any time by different ways. The physiological adaptations of the body represent the chronologic development of the response to stressors when their action is prolonged [4].

Since the muscle represents about ¾ of the body mass, a healthy muscular system is associated with the status of the other system of the body. Sick muscle system has harmful effects on human healthy and to its the capacity of interaction in this world. Skeletal muscle is a high plastic tissue that may be modified by use or disuse. Muscle composition, at chemical and structural levels, can be altered and these modification are related to the specific type of exercise that the organism is submitted. For example, the well know effect of strenght training is the hypertrophy of the muscle, which is associated to an increase in force production capacity. Strenght training may induce hypertrophy (muscle fiber enlargement)

and hyperplasia (increase in muscle fiber number) while endurance training promotes elevated muscle oxidative capacity (increase in mitochondrial number). All these adaptations are related to the health status of the body. On the other hand, the absence of exercise stimulus results in atrophy process and loss of functional capacity, marked by both impaired force production and metabolism of the muscle [5].

In this chapter, we focus on cell physiology and molecular biology of the muscle cells from a special point of view concerning to the stress response: the role of heat shock proteins (HSPs) in muscle.

2. Classical roles of heat shock proteins expression

Living organisms respond at cellular level to unfavorable conditions such as heat shock, and other stressful situations of many different origins, by a rapid, vigorous and transient acceleration in the rate of expression of specific genes: the heat shock genes. The products of these genes are commonly referred to as stress proteins or heat shock proteins (HSPs). Besides activation of heat shock genes, the expression of most other genes is inhibit as a result of stress. Thus, the stress intensity and duration leads to a perturbation of normal gene expression, which, if prolonged, can have drastic consequences for cells and system homeostasis [6, 7].

HSPs are highly conserved proteins in both eukaryotic and prokaryotic organisms and are expressed in many cell types including striated skeletal muscle. The first report about HSPs was documented by Ritossa [8, 9], after a serendipitous heat shock in salivary gland cells of *Drosophila buskii*, but heat shock proteins were only characterized later in 1974 [10]. Actually, HSPs are categorized in families according to their molecular sizes and include HSP110, HSP100, HSP90, HSP70, HSP60, HSP30 and HSP10 subclasses. In this chapter, the role of HSPs in muscle will be discussed in terms of the most studied (due to its evident high expression in mammalian cells under stress conditions) and conserved: the 70-kDa family (HSP70), which comprises a number of related proteins whose molecular weights range from 66 to 78 kDa. Many studies in human, rat and mice will be listed throughout the text, thus it is necessary to learn about the HSP70 isoforms that are encoded by a multigene family in each mammalian that will be listed below.

In humans, there are at least 13 distinct genes so far studied [11]. For the rationalization of the current nomenclature, human HSP70 genes (rat and mouse, also) have given the locus symbol HSPAx, where A defines members of HSP70 family and X designates the individual loci. In this sense, HSPA8 is the human gene that encodes a 73-kDa constitutive form of HSP70 (HSP73 or HSC70, the cognate form), while HSPA1A gene, located at the major histocompatibility complex (MHC) III region, encodes an inducible form (HSP72 or simply HSP70). In humans, but not in the rat or the mouse, there is an even higher inducible form (HSP70B') encoded by HSPA6 gene. Other representative members, besides mitochondrial (HSP75) and endoplasmic reticulum (HSP78) members of HSP70 family, are found in the intracellular space. While the constitutive form is expressed in a wide variety of cell types at basal levels (being only moderately inducible), the so-called inducible HSP70 forms (which

are barely detectable under non-stressful conditions) could be promptly synthesized under a condition of 'homeostatic stress', this being any 'homeostasis threatening' condition, such as heat, glucose deprivation, lack of growth factors and so forth. Habitually, research groups indistinctly use HSP70 as a unified term for both constitutive and inducible form. However, HSP70 is the preferable form to be used when one refers to the inducible HSP72 protein encoded by HSPA1A gene [12, 13]

In rodents, the Hsp70 family consists of at least nine members that differ from each other by the intracellular localization and expression pattern. Two of them, Grp78 and Grp75, are localized in the lumen of the endoplasmic reticulum and in the mitochondrial matrix, respectively, whereas the remaining seven HSP70s reside mostly in the cytosol. The only cytosolic HSP70 abundantly and constitutively expressed in all cells is Hspa8 (related to Human constitutive form HSP73). The related form for HSP72 in rodents are two proteins encoded by almost identical genes, *Hspa1a* and *Hspa1b*, termed collectively Hsp70i. As in humans, the expression of Hsp70i in rodents is low or undetectable in most "resting" normal cells and tissues, but it increases rapidly in a variety of stress conditions [14-16].

The heat shock response is regulated by high conserved cis-acting regions of the DNA (heat shock elements – HSE) and also by high conserved DNA associated trans-acting proteins named Heat Shock Transcription Factors (HSF) (Morimoto et al., 1992). While more simple organisms as insects (*Drosophila melanogaster)* and yeasts (*Saccharomyces cerevisiae)* have only one HSF, rodents have at least 2 HSF (HSF1 and HSF2) and humans have 3 isoforms of HSF (HSF1, HSF2 e HSF4) [17-20]. Possibly, more complex organisms could have used multiple HSF against different challenges during evolution. Comparing the structure of each isoform of HSF in one single species, the sequence of amino acids may be 40% identical, as HSF1 and HSF2 in mice [21, 22]. Comparing among species, the homology may be as great as 92%, as seen between HSF1 of human and rodents, or in HSF2 (95% homology between humans and rodents). Interestingly, HSF1 and HSF2 may be activated by distinct physiological phenomenon (Sistonen et al., 1992). While HSF1 and HSF2 are found in all kinds of cells, HSF4 is specifically for cells such as neurons or cardiac and skeletal muscle cells. It has also inhibitory function in heat shock response as negative regulator of HSPs expression [23, 24]. Additionally, in experimental models and cell culture procedures, is possible to identify differences in the activation of HSF1 and HSF2. The former is activated seconds after stress and this response is attenuated quickly, while the latter appears to present a latency period between the event and the response, but it remains activated for 72 h. This HSF different latency for activation suggest a cooperative role of HSF isoforms in cell protection [19] and that these genes are high conserved in nature [7].

In unstressed cells, HSP70 may bind to the regulatory protein HSF for prevention of the trimmer formation of HSF that is required for HSPs transcription. Under stressful conditions, the free HSP70 captures the denatured proteins and then dissociates from the HSP70-HSF complex allowing the formation of HSF trimmer, thus triggering a HSP70 production. Once synthesized, new HSP70 molecules may be involved in a variety of cellular processes and exert different functions [25, 26].

HSP70s are known to function as intracellular molecular chaperones that facilitate protein transport, prevent protein aggregation during folding and protect newly synthesized polypeptide chains against misfolding and protein denaturation. The molecular chaperones properties of such a protein allow them to assist the non-covalent assembly/disassembly of other macromolecular structures without being permanent components of such structures. Additionally, molecular chaperones assist the unfolded protein to achieve its single correct three-dimensional configuration (by still unknown mechanism it has evolved to generate this folded state), without becoming a constituent of the final folded protein [25, 26].

Most proteins destined for cell organelles are synthesized in cytosol and must cross one or more organelle membranes to reach their functional destination. For example, in the mitochondria, 95% of the proteins are made as precursor proteins in the cytosol and are mainly post translationally imported into the mitochondrial sub-compartments. In this situation, cytosolic HSP70 play an important role for maintenance of a transport-competent conformation of precursor proteins. The precursor protein is translocated in an unfolded state and are refolded later, sorted to their final destination and assembled into functional complexes [27].

The chaperone function of HSP70 includes the inhibition of the formation of nascent polypeptides. This inhibition is dose-dependent over a range of 0.1-0.4 nmoles of HSP70 and this effect is greater for the larger polypeptides. All these data suggest that high concentration of HSP70 can perturb the normal folding of nascent proteins, block cell growth and impair the cell viability. Then, these are reasons that may explain why the cells of human body have to carefully auto-regulate the levels of HSP70. Many characteristics and functions of HSFP70 are listed in table 1 and are discussed in this text.

Since the skeletal muscle is one of the most adaptable tissues of the body, every structural aspect of the muscle that can change in response to the stimulus challenge (or to its lack) may require the chaperon action of HSP70 at the molecular level. For example, major adaptations to (dis)use muscle, such as fiber type distribution, fiber diameter, myosin heavy chain profile and mitochondrial distribution, are factors that are associated to the up- or down-regulation of HSP70.

3. HSP70 function in the muscle

The ability of muscle cells to express HSPs (mainly HSP70s) represents a cytoprotection mechanism because HSP70 proteins share the same overall structure. They are composed of an actin-like N-terminal nucleotide binding/ATPase domain of 45 kDa, a substrate-binding domain (SBD) of approximately 15 kDa and a C-terminal domain of approximately 10 kDa that is involved in co-chaperone binding (HU et al, 2006). It is of note that N- and C-terminal domains have expressive relevance to antigen presentation, an important way by which HSP70s participate in immune responses. With this structure, HSP70 may act as a molecular chaperone inside the muscle cell: they facilitate protein transport; prevent protein aggregation during folding; protect newly synthesized polypeptide chains against misfolding and protein denaturation [28, 29].

Protein	Gene name	Human gene ID	Basal levels	Synthesis under stress condition	Cellular location	General functions and process	Specific functions
HSP72 (HSPA1A, HSPA1B)	HSPA1A, HSPA1B	3303, 3304	Low	Fast	Nucleus, nucleolus, citoplasm, cytoskeleton (Mainly in nuclear locus)	Protection against stress Participation in protein metabolism (protein degradation, folding and synthesis) Adaptation to stress Cellular development Cellular energy metabolism	Passes the newly synthesized, unfold protein to leading to folded protein Carries proteins for translocation into different cellular compartments Serve as cohort proteins to other proteins as a drug delivery vehicle Prevent aggregation of non native proteins Facilitate the functional restoration of denatured proteins or the degradation of irreversible damage proteins. Augmented glycolitic activity.
HSP73 (HSPA8)	HSPA8	3312	High	Slow	Distributed throughout the cell, but concentrated over mitochondria or in the nucleolus		

Table 1. Characteristics and functions of HSP70

Historically, studies about the effects of exercise on heat shock protein expression have been dedicated to HSP70 analysis in cardiac or skeletal muscle after exhaustive animal protocols (for review see Noble *et al.*, 2008) [30]. It has been demonstrated that 30 minutes after an exercise bout there is an increase in mRNA expression of muscle inducible form of HSP70 (HSP72) and that is a later heat shock response related to mRNA of constitutive form of HSP70 (HSP73) [31]. Similar response may be observed in humans, and are related to glycogen depletion and the muscle heating [32]. This acute heat shock response (intracellular HSP70 content) remained increased 24 hours after an acute exercise session, according to exercise load [33].

In humans, repeated bouts of eccentric exercise showed an impressive result in terms of HSP70 expression. Sets of Eccentric contractions contractions of the elbow flexors promote ~2-fold increase in HSP70 expression in biceps brachii. Four weeks later, the basal levels of HSP70 was reduced but the muscle still presents the heat shock response to exercise in the same magnitude but in less HSP70 content [34, 35]. Accompanying this effect, higher levels of both serum creatine kinase, soreness, lower levels of both relaxed arm angle and measured torque (indicators of muscle damage) occurs after the first bout, but the levels of these parameters are lower in the second bout. This may represents an association between muscular adaptations and the phenomenon called "acquired thermo tolerance" in terms of HSP70 expression. This study suggests that equivalent HSP70 response imply equivalent levels of stress in each bout and that may be an indicative that the heat shock response is a marker of muscle adaptation. Moreover, basal modification of HSP70 levels remains four weeks after the first bout of exercise and suggests that a single exercise challenge can promote deeper molecular adaptations in muscle cells [34].

Since the heat shock response is a prompt molecular adaptation to the stress condition, to localize the elements that contemplates this phenomenon in muscle is a interesting point of research. The sarcoplasmic reticulum contains microdomains that are involved in translation and processing of transcripts which encode proteins requiring compartmentalization to specific organelles within the myofiber [5]. In skeletal myofibers, ribosomes are localized to both the subsarcolemmal and intermyofibrillar cytoplasm. HSP70 has been shown to be concentrated in a subsarcolemmal fashion and it localizes to the nucleolus and myofibrils in response to stress condition. Although various modes of exercise can induce HSP70 expression, it is clear that it has a different pattern of heat shock response between slow and fast fibers. Slow and more oxidative fibers express greater HSP70 content in response to exercise possibly by preferential recruitment or a more sensitivity to temperature challenges. In this way, the muscle that have different localization of HSP70 mRNA in according to the type of the stimulus: exercise is different to heat treatment [36]. Exercise promotes a concentrated and punctuated perinuclear localization of HSP70 mRNA near the periphery of skeletal myofibers after exercise session (1 to 24h). This result represents that HSP70 proteins are prompt to response because HSP70 mRNA appears rapidly close to the nuclei that transcribes this gene. Diffuse HSP70 mRNA was also observed any time after exercise bout that represents a quickly cytoplasmic distribution of heat shock response proteins [36, 37]. Corroborating this discussion, Paulsen et al [38] showed that maximal eccentric exercise induces a 20 fold increase in HSP70 mRNA 24h after exercise and an increase about 200% in cytosolic HSP70 content.

The chaperone function of HSP70 is more than microscopic laboratory measurements research field. Muscle disuse results in muscular atrophy that is represented by decrease in muscle mass, fiber cross sectional area and total myofibrillar protein content. In this situation contractile protein breakdown exceeds protein synthesis. Moreover, in atrophied muscle there occurs an increase in the proportion of fibers containing the fast myosin heavy chain by transformation from the slow myosin heavy chain (MyHC-I/β) to the fast myosin heavy chain (MyHC-IId/x). As early as 18 h after muscle disuse and persisting for as long as for 18 days, it is possible it is possible to measure a decrease in HSP72 in soleus muscle [39]. Interestingly, previous heat treatment is a strategy to induce HSP70 expression in muscle and this molecular adaptation results in maintenance of muscle mass during 7 days period of immobilization [40]. In this way, HSP70 expression appears to have, not a full protective effect on muscle mass, fiber cross sectional area and total myofibrillar protein content, but a preventive effect on the decrease in MyHC-I/β and the increase in MyHC-IId/x induced during the atrophy process [41]. These evidences suggest that HSP70 can inhibit a key signaling pathway for atrophy in muscle cell preventing the muscular atrophy.

Heat treatment has also been tested in humans. Short wave diathermy therapy is a clinical strategy that means to increase deep heating of tissues with higher water content. This strategy may promote a 58%-increase in HSP70 expression in *vastus lateralis* [42]. It is possible that the previous heat treatment cannot reduce markers of muscle damage but it is able to reduce muscular pain, to preserve strength and to improve range of motion following eccentric contractions. Curiously, there is a gender difference in heat shock response in both basal and exercise-induced HSP70 levels, with men showing lower pre-exercise levels and an attenuated HSP70 response as compared to women's values. The gender difference may be explained by the effects of estrogen modulation on heat shock response [42].

If muscle disuse is a trouble, the reuse of the musculature may represent many stages of soreness. After immobilization, the reload process to the muscle implies in newest molecular adaptations. If a less-required muscle is submitted to a challenge, the HSP70 expression increases greatly (~200%) in the first two weeks of reload process and return to basal levels (above disuse levels) as early as in 8 weeks [39]. This effect is accompanied by increase in percentage of slow type I MyHC fibers (MyHC-I/β). Although many factors appear to be related to the down- and up-regulation of HSP70 function, the expression of this protein is closely related with the morphological and functional changes of muscle cells.

Although initially the HSP70s have been described essentially in studies that addressed molecular chaperone action of such proteins, HSP70s have also been studied as limiting of protein aggregation, facilitating protein refolding and maintaining structural function of proteins [43]. Intracellular HSP70s have further been demonstrated to be anti-inflammatory [44, 45], providing cytoprotection through anti-apoptotic mechanisms, inhibiting gene expression and regulating cell cycle progression [46].

Besides the now classical molecular chaperone action, the most remarkable intracellular effect of HSP70 is the inhibition of nuclear factor κB (NF-κB) activation, which has profound implications for immunity, inflammation, cell survival and apoptosis. Indeed, HSP70 blocks

NF-κB activation at different levels. For instance, HSP70 inhibits the phosphorylation of inhibitor of κB (IκBs), while heat-induced HSP70 protein molecules are able to directly bind to IκB kinase gamma (IKKγ) thus inhibiting tumour necrosis factor-α (TNFα)-induced apoptosis [47, 48] . In fact, the supposition that HSP70 might act intracellularly as a suppressor of NF-κB pathways has been raised after a number of discoveries in which HSP70 was intentionally induced, such as the inhibition of TNFα-induced activation of phospholipase A2, the suppression of inducible nitric oxide (NO) synthase (iNOS, encoded by NOS-2 gene) expression paralleled by decreased NF-κB activation. Hence, HSP70 is anti-inflammatory per se, when intracellularly located, which also explains why cyclopentenone prostaglandins (cp-PGs) are powerful anti-inflammatory autacoids [49, 50].

Another striking intracellular effect of HSP70 is the inhibition of apoptosis. Caspases form an apoptotic cascade by the intrinsic pathway, characterized by the release of mitochondrial pro-apoptotic factors into the cytosol, while stimulation of cell surface receptors triggers the extrinsic pathway by external signaling factors that may induce the apoptotic process. The inhibitory potential of HSP70 over apoptosis occurs via many intracellular downstream pathways (e.g. JNK, NF-κB and Akt), which are both directly and indirectly blocked by HSP70 either, besides the inhibition of Bcl-2 release from mitochondria. Together, these mechanisms are responsible for HSP70 anti-apoptotic function in cells under stress conditions [51-53].

These intracellular effects of HSP70 are closely related to aging and disuse (or both, in a synergic way) effects on muscle wasting, because there is comprehensive evidence that NF-κB activity is increased during disuse and is required for muscle atrophy. NF-κB activation is actually decrease in the first week of immobilization but it is increased in longer atrophy process (by 3-fold) and aged disused muscle (5-fold increase), both reversible effects with HSP70 overexpression that inhibit NF-κB activity owing to increasing the levels of IκBα that are available to bind and to retain NF-κB proteins in cytosol [40].

In terms of metabolic function, increased HSP70 protein expression (~50%) by heat treatment, muscle-specific transgenic over expression, or pharmacological means can protect against diet- or obesity-induced hyperglycemia, hyperinsulinemia, glucose intolerance, and insulin resistance. This protection was tightly associated with the prevention of JNK phosphorylation, another role for HSP70 in the blocking of inflammation [54].

4. Muscle activity and HSP70, eHSP70 and cytokines

Cytokines are intracellular signaling molecules, typically proteins or glycoproteins, that mediate various aspects of cell function, including proliferative and adaptive responses. Cytokine signaling is essential for a coordinated inflammatory response. Diseases related to inflammatory processes as cancer, congestive heart failure, AIDS, sepsis and arthritis often lead to muscle catabolism and loss of muscle function, and this effects are attributed to circulating cytokines. On the other hand, exercise is known to alter immunological function in health individuals and this adaptation also is related to altered cytokine levels [55].

Some cytokines are more closely related to exercise challenge. Circulating TNF-α (tumor necrosis factor – α) may promote cellular responses mediated by two receptors located on cell surface, the 55 kDa TNF-receptor 1 and the TNF-receptor 2. The chronic interaction of this cytokine with its receptors resulted in catabolic response, as loss of muscle mass and contractile dysfunction. TNF- α promotes loss of muscle protein associated to oxidative stress signaling that culminates in muscle wasting mediated by the NF-κB activation, a redox sensitive transcription factor. The impaired muscle function induced by TNF- α also may occur without changes in muscle mass [56]

Exercise-associated muscle damage initiates the inflammatory cytokine cascade. Strenuous exercise increases plasma levels of TNFα, IL-1, IL-6, IL-1 receptor antagonist (IL-1ra), TNF receptors (TNFR), IL-10, IL-8, and macrophage inflammatory protein-1. Exercise induces immune changes and also alters neuroendocrinological factors including catecholamines, growth hormone, cortisol, β-endorphin, and sex steroids. It is generally assumed that the "brain–immune" axis also exists during stress. Release and/or expression of enkephalins can be regulated by different factors such as stress, exercise and cytokines [55, 57].

IL-6 is generally considered a pro-inflammatory cytokine released from immune cells and reaching higher levels in the circulation and inside the muscle. However, muscle contraction during exercise is a signal for IL-6 release from the muscle. IL-6 increases ~100-fold after a marathon race and the increase was tightly related to the duration and intensity of the exercise. IL-6 is produced in the skeletal muscles in response to exercise and it has growth factor abilities and contributes to the anti-inflammatory effect of exercise. Interestingly, exercise, IL-6 and HSP70 have particular relationship: exercise training increases IL-6 response to immune related challenge (LPS treatment) and IL-10 plasma concentration; IL-6 can induce HSP70 expression but the absence of IL-6 during exercise do not attenuate the increase of HSP70 expression by exercise; in sedentary, the absence of IL-6 blunted HSP70 response in skeletal muscle after a immune challenge (LPS treatment); and the absence of IL-10 (an anti-inflammatory cytokine produced during exercise) increased the levels of IL-6 after the same immune challenge These data suggest that there are different pathways that leads to IL-6 and HSP70 up-regulation, with and without exercise stimulus [44, 45, 58-61]. These cytokine signaling and HSP70 expression effects on muscle are summarizing in Figure 1.

Physical exercise has many effects on the Central Nervous System (CNS), much more than mood influence. Peripheral signals generated during and after an exercise session, such as IL-6 and IL-10, decrease endoplasmic reticulum stress markers at hypothalamic level, an effect related to the decrease in NF-κB activation. The processes of building certain behaviors and control of them can be analyzed under the optics of neuroimmunomodulation. The expression of 'sickness behavior' can be induced by immune modifications and immune capacities that are associated with distinct behavior in mammals. In this sense, it is clear the participation of mediators including TNF-α, interleukin-1β (IL-1 β), and IL-6 in the CNS. For instance, the release of skeletal muscle-derived IL-6 into the blood is the most remarkable alteration in cytokine pattern observed

during exercise so that IL-6 is now considered as an exercise factor, a 'myokine' [60], not just an inflammatory mediator. Additionally, as previously hypothesized, [61] the exercise-evoked IL-6 may also act on the CNS to induce the fatigue sensation. In other words, the skeletal muscle must be considered as an auxiliary endocrine organ that interacts with the immune system and CNS, so that IL-6 is a robust exercise marker. Myokine signals are correlated with sensation of fatigue, and may be inducer of sleep or illness response and pyrogenic behavior.[62-67].

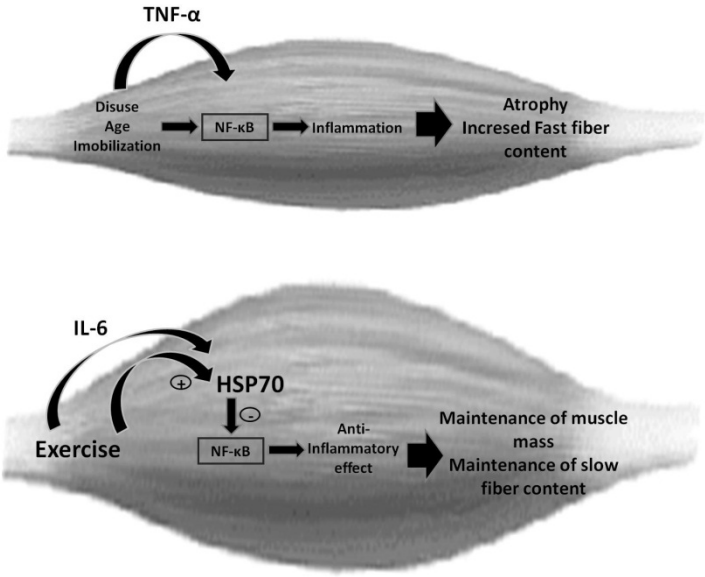

Figure 1. The HSP70 role in inhibition of NF-κB induced atrophy

More recently, however, it has been demonstrated that the presence of HSP70s in the circulation (extracellular HSP70, eHSP70) in response to exercise [32, 44, 68-70]. Since exercise is able to induce high concentrations of HSP70s in both muscle and plasma, the most obvious hypothesis was, primarily, that skeletal muscle should be the releaser of HSP70 during exercise. However, further studies have revealed that this is not the case, at all. The lack of evidence supporting the proposition that the muscle could be the major source of circulatory eHSP70 precluded the 'muscle hypothesis' and suggested that other tissues/cells should be responsible for the increase of eHSP70 in the circulation. In the early phase after high-intensity exercise, eHSP70 is elevated in peripheral blood.

Once HSP70 protein release from muscle to extracellular fluid could eventually happen by lysis process, and considering that the lysis of muscle fiber occurs only under severe cellular stress condition, the presence of eHSP70 during moderate exercise was found to be unfeasible. Though it had been shown that both the intensity and duration of exercise have effects in plasma[71] and muscle[33] HSP70 concentration, this rise in circulating levels of HSP70 precedes, however, any gene or protein expression of HSP70 in skeletal muscle,[32, 72] which

is another strong argument against the 'muscle hypothesis'. Afterwards, eHSP70 blood concentration returns to the lower basal levels as soon as 2 h after the end of the physical effort, remaining practically undetectable for 24h. Similarly to the cytokines released by immune cells during exercise, serum eHSP70 concentration does rise after exercise sessions, mainly because of the contribution of lymphocytes [73]. As a corollary, lymphocyte-derived HSP70s may interplay with CNS to induce the state of 'fatigue behavior' activation [45]. Then, the equilibrium of immune signals during exercise is required to maintenance of the homeostasis and this equilibrium may be observed by several markers, listed in the Figure 2 in relation to the degree of exercise or disease challenge.

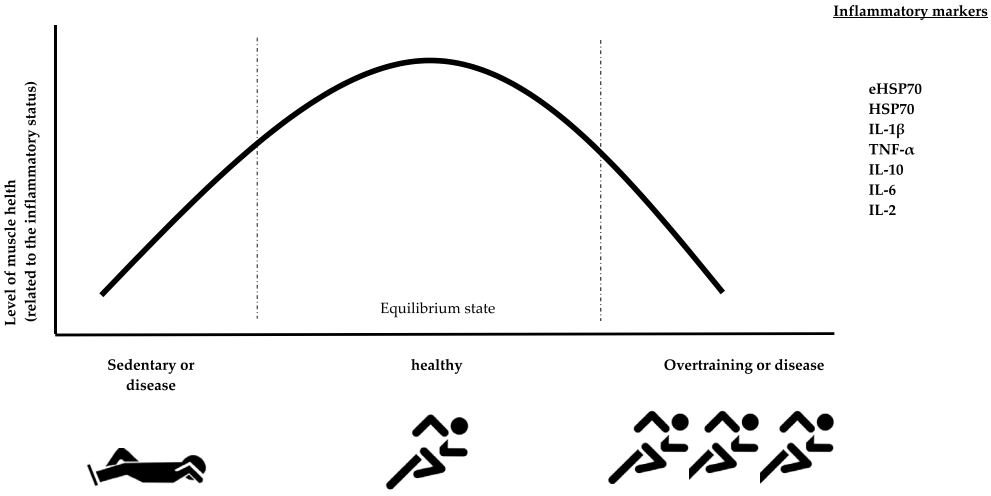

Figure 2. Relation between the degree of exercise or disease challenge and inflammatory markers: The proposed markers may represent both the inflammatory status and muscle status of health. In this way, the figure represents the hypothesis that there is an equilibrium state of many markers in health and a disequilibrium in sedentary, disease and overtraining situations. These markers include extracellular/intracellular HSP70 ratio hypothesis and many others cytokines.

There are many diseases related with higher levels of eHSP70, suggesting that serum levels of this proteins may be considered a novel important biomarker. Whereby health people have low plasmatic levels of eHSP70, the association of these proteins with illness, disease progression and mortality was hypothesized, as well as longevity and health parameter status were attributed to this lower concentration. On the other hand, a rise 3.7-fold eHSP70 circulating levels in critically ill patients was correlated with less hospital treatment period [74] and death [75].

The increase in eHSP70 during the exposure to stresses has also been demonstrated to be the result of the activation of the sympathetic nervous system via alpha-adrenergic receptors leading to eHSP70 export and increased eHSP70 serum concentration[76, 77]. Thus, even though the necrotic cell death might result in the appearance of HSP70 within the extracellular milieu, an increasing number of studies suggest that this is not the major

rule but, on the contrary, physiological effectors (e.g. fever, hypoglycemia and sympathetic stimulation) are the true excitatory signals for the eHSP70 exocytotic pathway, which suggests that highly conserved evolutionary responses are tightened to eHSP70 production, meaning that extracellular HSP70 response may have had an important evolutive role.

The interaction of cytokines or eHSP70 with the complexes of toll-like receptor (TLR2 and/or TLR4) acts as inflammatory signal to cells of the innate immune response (macrophage/dendritic cells/neutrophils). Under stimulation of TLRs, eHSP70 signalizes to the increase of the signal transduction of NF-κB dowstream pathways. Asea and co-workers have shown that eHSP70 induces NF-κB activation and the production of inflammatory cytokines in a process that requires CD14, in addition to TLR2 and TLR4 that are expressed in muscle cell surface [78-81].

By definition, cytokines are proteins secreted by cells with regulatory effects on other cells. Therefore, in addition to its function as an intracellular molecular chaperone, HSP70 in the extracellular milieu acts as a powerful cytokine, affecting the functional properties of immunocompetent cells. This dual role, as both a chaperone and cytokine, helps to elucidate recent findings indicating that heat-shock proteins can be potent adjuvant for many inflammatory related diseases [79].

5. Conclusion

In summary, HSP70s have physiological proprieties that are involved in maintenance of muscle muscle function by the interaction with molecular entities inside the skeletal muscle cell and also by via cell surface receptor. Exercise-induced increase in HSP70 expression and eHSP70 concentration have important role in the regulation of the inflammatory pathways that can be activated during high intensity exercise as well as in the course of atrophy process.

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Mechanical Response

Chapter 6

Evidence for the Essential Role of Myosin Head Lever Arm Domain and Myosin Subfragment-2 in Muscle Contraction

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Additional information is available at the end of the chapter

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1. Introduction

It has been well established that muscle contraction results from relative sliding between the thick and thin filaments [1,2]. Since the myofilament sliding is coupled with ATP hydrolysis, physiological function of muscle is to convert energy derived from chemical reactions into mechanical work and heat. The thick filament consists mainly of myosin, which is a large molecule (MW 450,000) with two pear-shaped "heads" and a "rod" of 156nm long. As illustrated in Fig.1A, a myosin molecule is split by tryptic digestion into two parts: (1) a rod of 113nm long, known as light meromyosin (LMM), and (2) the rest of the myosin molecules, containing the two heads and a rod of 43nm long, known as heavy meromyosin (HMM). HMM is further digested into two separate heads, known as subfragment-1 (S-1), and the rod, known as subfragment-2 (S-2). When myosin molecules polymerize to form the thick filament, LMM aggregates to constitute the filament backbone, which is polarized in opposite directions on either side of the central region, while the S-1 heads extend laterally from the filament backbone with an axial interval of 14.3nm, except for the central region where the S-1 heads are absent and called bare zone (Fig.1B). The S-2 rod is believed to serve as a "hinge" between the S-1 head and the filament to enable the head to swing away from the filament.

On the other hand, the thin filament consists primarily of two helical strands of globular actin monomers (G-actin, MW 41,700), which are wound around each other with a pitch of 35.5nm. The axial separation of G-actin in the filament is 5.46nm (Fig.1C). In vertebrate skeletal muscle, the thin filament also contains tropomyosin and troponin. The tropomyosin molecule is rod shaped, and lies in the grooves between the G-actin strands, while troponin

molecule is located in every pitch of the strands. In relaxed muscle, interaction between the S-1 heads and the thin filament is inhibited by tropomyosin. When Ca^{2+} binds to troponin, it removes the inhibitory effect of tropomyosin to start muscle contraction [3].

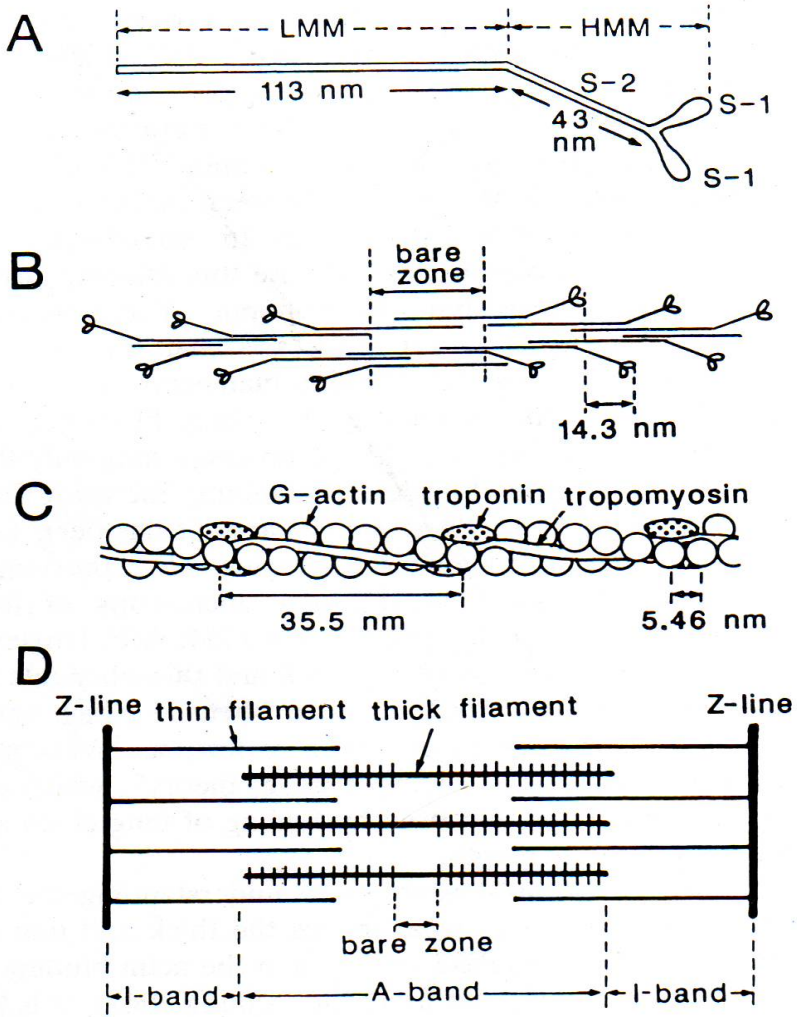

Figure 1. Structure of the thick and thin filaments and their arrangement within a sarcomere. **A** Diagram of a myosin molecule. **B** Longitudinal arrangement of myosin molecules in the thick filament. **C** Structure of the thin filament. **D** Longitudinal arrangement of the thick and thin filaments within a sarcomere. Note that the half-sarcomere is the structural and functional unit of muscle.

A skeletal muscle fiber is composed of longitudinally repeated structural units, called sarcomeres, which are bounded by Z-lines. As diagrammatically shown in Fig.1D, the thin filaments extend in either direction from the Z-line to penetrate in between the thick

filaments, located centrally in each sarcomere [3]. The region containing only the thin filaments is called the I-band, while the region containing the thick filaments as well as part of the thin filaments is called the A-bands. It has been firmly established that the filament lengths remain constant during active contraction and passive stretch of muscle fibers, based on (1) light microscopy of muscle fibers and myofibrils [1,2]; (2) electron microscopy of the filaments [4]; (3) X-ray diffraction of muscle fibers, in which various periodicities of the filaments can be measured [5]. These findings constitute evidence for the sliding filament mechanism of muscle contraction, which appears in every textbook in physiology.

Thus, the central problem in understanding molecular mechanism of muscle contraction is: what makes the thick and thin filaments to slide past each other? Since the ATPase activity and the actin binding site are located in the S-1 heads of myosin molecule, it is generally believed that the myosin S-1 heads, extending from the thick filaments towards the thin filaments, may play an essential role in the chemo-mechanical energy conversion taking place in contracting muscle.

2. Theories of myofilament sliding producing muscle contraction

Since the lengths of the thick and thin filament remain unchanged before,during and after the myofilament sliding, i.e. muscle contraction, it seems natural to consider that the myofilament sliding is caused by cyclic formation and breaking of linkages between the S-1 heads on the thick filaments and the corresponding sites on the thin filaments. The cyclic interaction between the S-1 head and the thin filament is obviously coupled with ATP hydrolysis. Most theories about mechanism of muscle contraction have been based on this idea.

2.1. Attachment-detachment cycle between the S-1 head on the thick filament and the sites on the thin filaments

Fig. 2 shows diagrams illustrating hypothetical attachment-detachment cycle between the myosin S-1 head and the corresponding site on the thin filaments, put forward by H.E.Huxley [6]. In each diagram, three rectangular-shaped S-1 heads extend from the thick filament upwards to face the sites on the thin filament, represented by small rectangular projections. Left S-1 head first attaches to the site on the thin filament, which happened to be in its close vicinity (top), changes its configuration to move the thin filament to the right (arrow, middle), and then detach from the site on the thin filament (bottom). As the result, another site on the thin filament comes in close vicinity of right S-1 head, which then starts the cycle again. Axial spacing of the S-1 heads on the thick filament differs from that of the sites on the thin filament, so that the attachment-detachment cycle takes place asynchronously. Up to the present time, the attachment-detachment cycle shown in Fig.2, still constitutes the framework of most contraction models at the molecular level. The most crucial step of the attachment-detachment cycle is, of course, conformational changes of the S-1 head attached to the thin filament shown in the middle diagram.

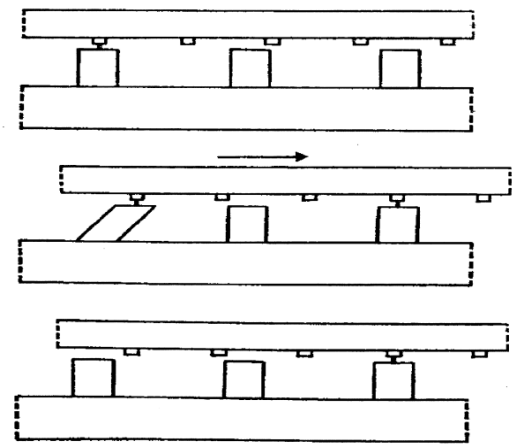

Figure 2. Diagrams of putative attachment-detachment cycle between the S-1 head, extending from the thick filament, and the corresponding site on the thin filament. For explanations, see text.

2.2. Actomyosin ATPase reaction

Molecular mechanism of muscle contraction can be studied biochemically by examining ATPase reaction steps of actin-myosin complex (actomyosin) in solution. To relate the enzyme kinetics of actomyosin in solution, the following limitations should be kept in mind: (1) the fundamental function of muscle to convert energy from chemical reactions into mechanical work can not be studied on actomyosin in solution; (2) no theories exist to directly relate the enzyme kinetics of actomyosin to the cyclic interaction between the thick and thin filaments in muscle; and (3) to study the enzyme kinetics in solution, fragments of myosin molecule (HMM or S-1) should be used at concentrations <several μM, while the effective concentration of myosin molecule and myosin S-1 head in muscle are >100μM.

The most probable sequence of actomyosin ATPase reaction taking place in contracting muscle is shown in Fig.3 [7,8]. M and A represent the myosin S-1 head on the thick filament and the actin monomer on the thin filament, respectively. The reaction cycle includes attachment of A to, and its detachment from M, thus providing a simple correspondence with the attachment-detachment cycle between actin and myosin in the Huxley contraction model shown in Fig.2, the above actomyosin ATPase reaction shceme is, therefore, believed to be applicable to the attachment-detachment cycle between the S-1 heads and the thin filaments in muscle.

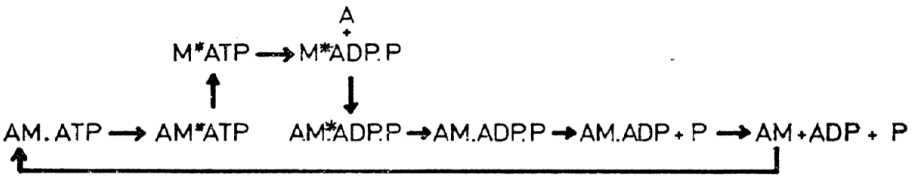

Figure 3. The most probable sequence of actomyosin ATPase reactions taking place in contracting muscle.

2.3. The myosin S-1 head tilting model

As illustrated in Fig.2, the S-1 head, attached to the thin filament, should undergo structural changes to cause relative sliding between the thick and thin filaments. Although a number of attempts have been made up to the present time using a variety of experimental methods, the question, as to what makes the filaments slide, is not yet fully answered. In 1977, A.F.Huxley and Simmons presented a contraction model, which was central in the field of muscle physiology over many years [9](Fig.4). The essense of this model is summarized as follows: during muscle contraction, each S-1 head first attaches to the thin filament (A→B), changes its angle of attachment to the thin filament from 90° to 45°, while attached to the thin filament (B→C), thus producing a unitary step for filament sliding, and then detach from the thin filament (D). In this contraction model, the S-2 only serves to transmit force generated by the "tilting" S-1 head to the thick filament backbone. A number of investigators made experiments to prove the "S-1 head rotation", including the use of fluorescent ATP and ADP analogs [10] and spin labels [11] attached to the S-1 head, with results that the change in S-1 head orientation was very limited during muscle contraction. Time-resolved X-ray diffraction studies on contracting muscle also could not detect any appreciable changes in the equatorial reflections in response to a quick decrease in muscle length, which was expected to synchronously rotate the massive S-1 head [12,13]. Thus, the myosin S-1 head tilting model was found to be inconsistent with the experimental observations.

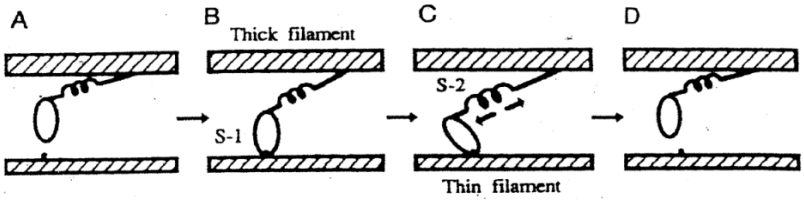

Figure 4. Diagram showing the attachment-detachment cycle between the S-1 head and the thin filaments, presented by A.F. Huxley and Simmons [10]. For explanations, see text.

Since the S-1 head tilting model is not supported experimentally, the S-1 head structural changes are considered to be limited within a small region in the myosin molecule.

2.4. The swinging lever arm hypothesis

The X-ray S-1 head crystal structure was first obtained by Rayment et al.[15] on chicken skeletal muscle. As shown in Fig.5, the S-1 head structure is tadpole-like in shape, with an elongated head containing the catalytic domain (CD) consisting of 25K (green), 50K (red) and part of 20K (dark blue) fragments of myosin heavy chain, and a tail, i.e. the lever arm domain (LD) consisting of the rest of 20K fragment and the essential (ELC, light blue) and the regulatory (RLC, magenta) light chains. The CAD and the LD are connected via the converter domain (COD). In the intact myosin molecule, the LD is connected to the thick filament via the myosin S-2. Attempts have been made to study possible nucleotide-dependent structural changes of the S-1 head to obtain insight into the mechanism of

muscle contraction. For this purpose, the S-1 head is truncated at its tail except for the COD, thus eliminating the LD including the ELC and the RLC. The truncated S-1 is nearly globular in shape, and is easy to crystallize. It is regarded as "minimal motor" to obtain information about muscle contraction mechanism, though the validity to eliminate and ignore possible function of the LD and the S-2 in muscle contraction is not proved up to the present time.

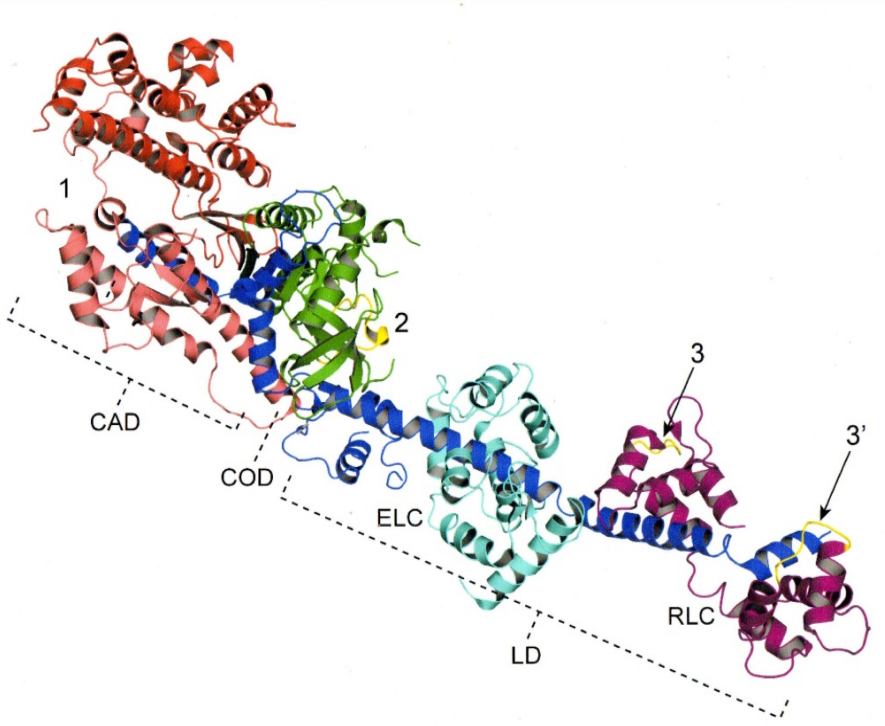

Figure 5. Myosin S-1 head structure [15]. For explanations, see text. Location of peptides around Lys 83 and that of two peptides (Met 58~Ala 70 and Leu 106~Phe 120) in the LD are colored yellow. Numbers 1, 2 and 3,3' indicate approximate regions of attachment of antibodies 1, 2 and 3, used by Sugi et al., respectively [14]. For further explanations, see text. Figure prepared by using software PyMOL (http://pymol.sourceforge.net.).

The crystal structures of truncated myosin obtained from a slime mold *Dictylostelium* have been studied with various ATP analogs, including ADP • BeF_3, ADP • AlF_4, and ADP • vanadate, and it has been shown that the truncated S-1 takes two different structures depending on the kind of ATP analogs; between the two states, the COD rotates by about 60° [for a review, see15]. Based on this and other results, it has been proposed that the power stroke of the S-1 head, causing myofilament sliding, results from active rotation of the CD around the COD [15], utilizing chemical energy of ATP hydrolysis taking place in the CD. This is the swinging lever arm hypothesis, which now appears in many textbooks in physiology and biology.

It is not certain, however, whether the above nucleotide-dependent structural changes of the S-1 head actually work in muscle contraction or not by the following reasons: (1) It is not clear that the observed rotation of the COD generates torque large enough to cause the filament sliding when the COD is connected to the thick filament via the LD and S-2; (2) It seems possible that the rotation of the COD is an artifact arising from close packing of the S-1 in the crystal, that may make each S-1 in a condition completely different from that in muscle; (3) It seems also possible that the ATP analogs used do not actually mimic intermediate compounds of ATP hydrolysis in muscle; and (4) The hypothesis completely igonores possible roles of the LD as well as the S-2.

2.5. Power and recovery strokes of the S-1 head coupled with ATP hydrolysis

Fig. 6 illustrates the most plausible attachment-detachment cycle between the S-1 head (M), extending from the thick filament and actin monomer (A) in the thin filament, based on the actomyosin ATPase reaction shown in Fig.3 [7,8]. First, M in the form of complex, M・ADP・Pi , attaches to A (A), and exerts a power stroke, associated with release of Pi and ADP (from A to B). After the end of power stroke, M remains attached to A (B). Upon binding with ATP, M detaches from A, and exerts a recovery stroke, associated with reaction, M・ATP → M・ADP・Pi (from C to D). Then, M・ADP・Pi again attaches to A (from D to A), and the cycle is repeated. In this diagram, both the power and the recovery strokes of M is supposed to result from the swinging lever arm mechanism, so that M does not change its angle of attachment to A throughout the whole cycle, while it swings around a pivot (COD, represented by a small circle) forward and backward. The LD is located between M and the pivot, simply serving as a lever arm.

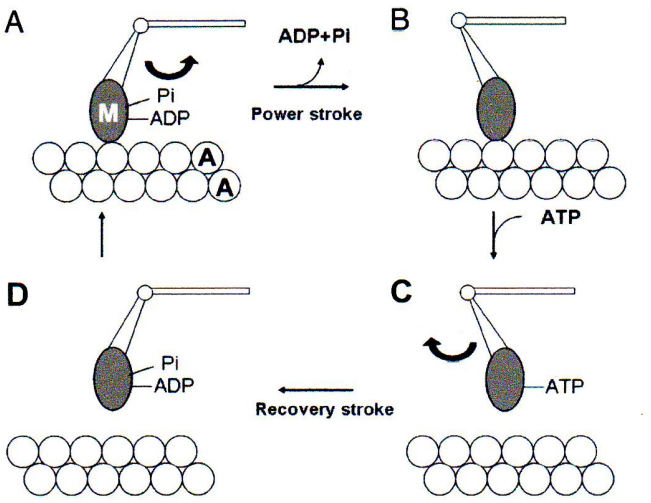

Figure 6. Diagram showing the attachment-detachment cycle between myosin S-1 head (M), extending from the thick filament, and actin monomer (A) in the thin filament, based on the actomyosin ATPase reactions [16]. For explanations, see text.

It is understandable that crystallographists use truncated S-1 head because of easiness in crystallizing it. Another reason for their ignorance of the LD and the S-2 in considering the mechanism of muscle contraction may come from development of so-called in vitro motility assay experiments, in which fluorescently labeled actin filaments are made to slide over myosin molecules or their proteolytic fragments such as HMM and S-1, fixed on a glass surface in the presence of ATP [17]. Especially, the fact that even the S-1 alone can generate force on actin filaments [18] seems to have given muscle investigators a belief that only the S-1 head is important in producing muscle contraction.

Fig.7 is a more realistic diagram showing the myosin S-1 head power stroke based on the swinging lever arm mechanism, in which the myosin S-1 head power stroke is accompanied by swing of the SD around the COD and also swing of the LD around the boundary between the LD and the S-2, while the CD structure remains unchanged before (solid line) and after (broken line) the power stroke. We think that there is no reason to ignore the LD-S-2 boundary in considering the mechanism of the myosin S-1 head power stroke. In the next section, we will present experimental evidence for the essential role of the LD and the S-2, as well as evidence for non-essential role of the COD.

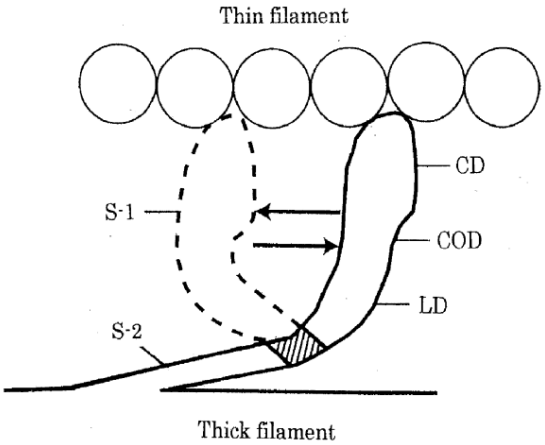

Figure 7. Diagram showing the structural changes of the myosin S-1 and S-2 before (solid line) and after (broken line) the power stroke. Note that the S-1 swings around the boundary between the S-1 LD and the S-2 (shaded area) in addition to the CD swing around the COD).

3. Evidence for the essential role of the S-1 head lever arm domain (LD) and the myosin subfragment-2 (S-2) region in muscle contraction

Our experiments concerning the essential role of the myosin S-2 started in 1992 when one of us (H.S.) was asked by the late Professor Harringon of Johns Hopkins University to work with him using a polyclonal antibody directed to the myosin S-2 (anti-S-2 antibody). At that time, Harrington had a unique idea that, in addition to the S-1 head power stroke, shortening of the S-2 region resulting from α-helix to random coil transition within a limited part of S-2 [19,20].

3.1. Effect of anti-S-2 antibody on the contraction characteristics of Ca^{2+}-activated rabbit psoas muscle fibers

Single glycerol-extracted muscle fibers, prepared from rabbit psoas muscle, were maximally activated with 10^{-4}M Ca^{2+} in contracting solution before and at various times after administration of anti-S-2 antibody (1.5mg/ml). Force-velocity (P-V) curves were determined by applying ramp decreases in force at the plateau of Ca^{2+}-activated isometric force with a servo-motor. Muscle fiber stiffness was measured by applying small sinusoidal length changes (1 kHz, peak-to-peak amplitude ~0.1% of fiber length) and recording resulting force changes [21].

The magnitude of Ca^{2+}-activated isometric force in the fibers decreased with time in the presence of antibody, while the maximum unloaded velocity of shortening V_{max} remained unchanged, as shown by the P-V curves in Fig.8A [21]. If the velocity values are replotted against forces expressed relative to steady forces, the P-V curves were found to be identical (Fig.8B). Since muscle fiber stiffness changed in parallel with force (Fig.9), the decrease in force is due to decrease in the number of myosin S-1 head involved in force generation, and myosin S-1 heads that stop interacting with the thin filament do not provide internal resistance against fiber shortening [21].

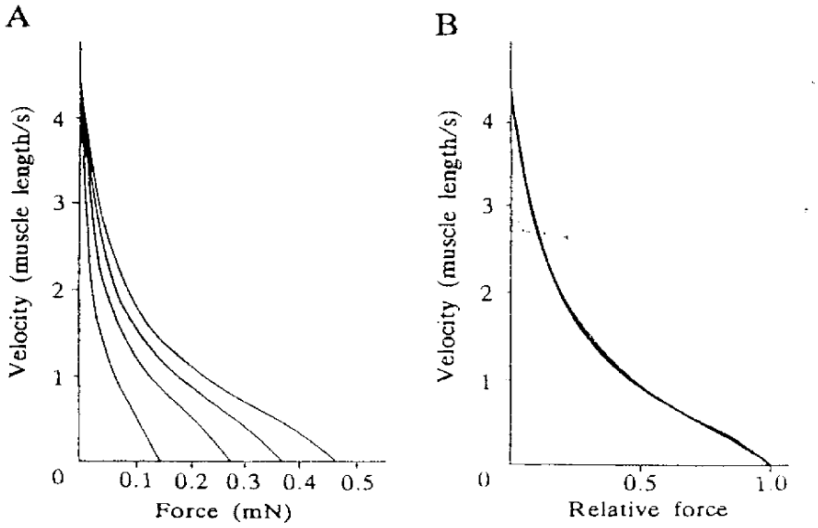

Figure 8. Effect of anti-S-2 antibody on P-V relation in a Ca^{2+}-activated single muscle fiber. (A) P-V curves obtained before (control) and 30, 60, and 90min after administration of anti-S-2 antibody. Both velocities and forces are expressed in absolute values. Note that the maximum shortening velocity remains unchanged despite marked reduction of isometric force. (B) The same P-V curves in which forces are expressed relative to their respective steady forces. Note that the curves are identical in shape [21].

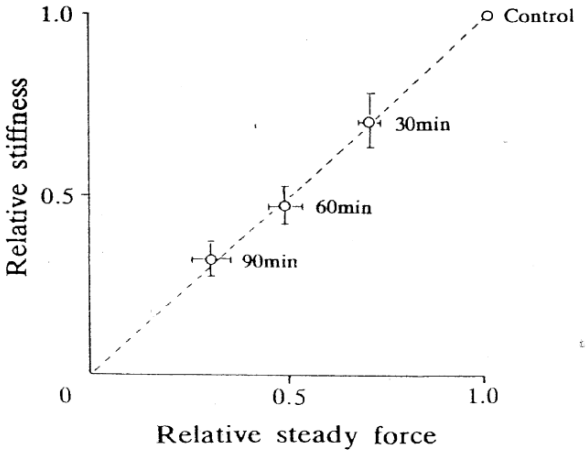

Figure 9. Stiffness versus force relation at steady Ca^{2+}-activated isometric forces of single muscle fibers before (control) and 30, 60 and 90min after administration of anti-S-2 antibody (1.5mg/ml). Each data point represents the mean of seven different experiments. Vertical and horizontal bars indicate SD of stiffness and force, respectively [21].

3.2. Effect of anti-S-2 antibody on MgATPase activity of Ca^{2+}-activated muscle fibers

MgATPase activity of Ca^{2+}-activated muscle fibers was recorded by decrease of NADH during cleavage of ATP [21]. A small fiber bundle consisting of 2—3 fibers was mounted in the sample compartment of a dual-wavelength spectrophotometer (model 156, Hitachi) with a sample monochrometer at 340nm and a reference monochrometer at 400nm. Examples of simultaneous recordings of MgATPase activity and isometric force development in the fibers are shown in Fig. 10. It was found that the MgATPase activity, judged from the slope of the ATPase records, did not change appreciably even when the force development was completely eliminated, indicating a complete dissociation of force development from MgATPase activity. This implies that, anti-S-2 antibody inhibits Ca^{2+}-activated force development by impairing the function of the S-2 region in producing the myosin head power stroke.

A question arises as to how the chemical energy of ATP hydrolysis, taking place in the myosin head CD can be transmitted to the S-2 region, distant from the CD region. Kobayashi et al. [22] have presented results suggesting that the myosin head CD can communicate with the myosin S-2. If anti-S-2 antibody is applied to the fiber after development of the isometric force, both the force and the stiffness decrease in parallel with each other with time. If, on the other hand, anti-S-2 antibody is applied to the fiber after development of rigor force in the absence of ATP, it shows no effect on both rigor force and stiffness. If it is assumed that the S-2 participates not only in Ca^{2+}-activated force development in the presence of ATP, but also in rigor force development in the absence of

ATP, then these results suggest that anti-S-2 antibody binds with the S-2 when the myosin head CD is interacting cyclically with the thin filament, but not when the CD stops cyclic interaction with the thin filament due to permanent rigor linkage formation.

Meanwhile, our recent unpublished experiments have indicated that a monoclonal anti-LD antibody, which attaches to the regulatory light chain close to the LD-S-2 boundary, inhibits Ca^{2+}-activated muscle fiber contraction. This, together with the inhibitory effect of anti-S-2 antibody on muscle fiber contraction, strongly suugests the essential role of the LD-S-2 boundary in muscle contraction.

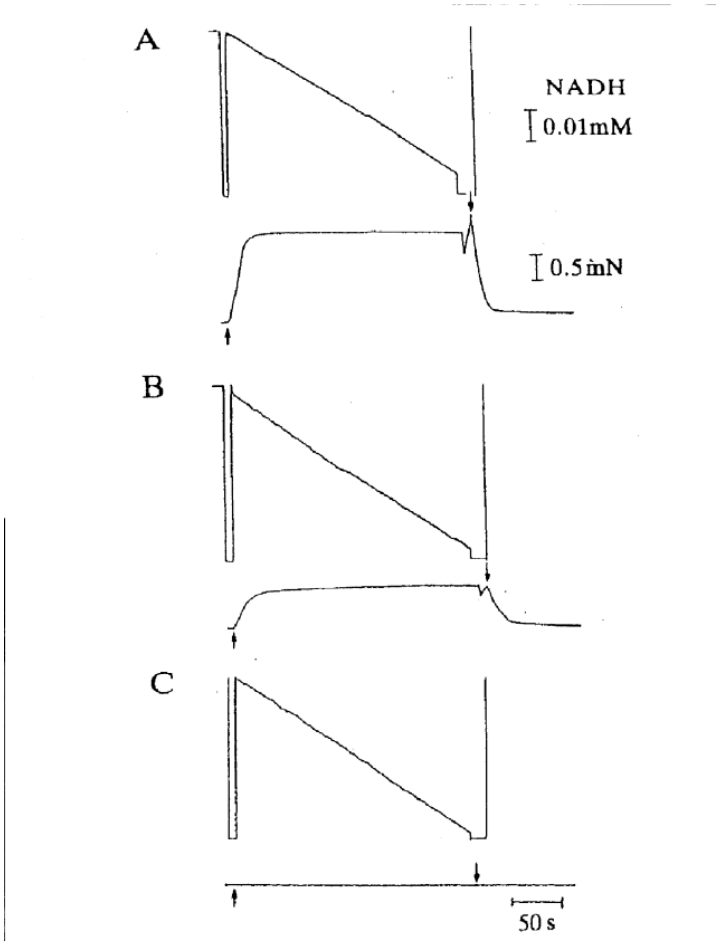

Figure 10. MgATPase activity (upper traces) and Ca^{2+}-activated isometric force (lower traces) in a small fiber bundle, before (A), at 100min (B) and at 150min (C) after application of antibody. Note that the slope of ATPase records does not change appreciably even when the force is reduced to zero. Times of application of contracting and relaxing solutions are indicated by upward and downward arrows, respectively [21].

3.3. Evidence against the essential role of the myosin head converter domain (COD) in muscle contraction

Muhlrad et al. [23] reported that chemical modification (trinitrophenylation) of reactive lysine residue (RLR), located close to the myosin head converter domain (COD) inhibited both actin-activated ATPase activity of RLR-modified S-1 head and in vitro actin filament sliding over RLR-modified myosin heads. They interpreted these results as being due to steric clashes between the modified RLR and the CVD structure to inhibit the myosin head power stroke as well as the ATPase activity in the myosin head CD. Their results seem to indicate the essential role of myosin head COD in muscle contraction,

Using the gas environmental chamber, which enables us to study dynamic structural changes of hydrated biomolecules retaining their physiological function, we succeeded in recording ATP-induced movement of myosin S-1 heads (mean amplitude, ~6nm) extending from hydrated synthetic thick filaments [16,24]. To position-mark individual S-1 heads in unstained filament samples, we used a monoclonal antibody directed to junctional peptide between 50K and 20K fragments of myosin heavy chain (antibody 1 in Fig.5).

Also using the gas environmental chamber, we could record ATP-induced movement at three different parts within individual S-1 heads with three different site-directed antibodies; antibody 1, antibody 2 to reactive lysine residue (RLR) located close to the COD, and antibody 3 to two peptides in the regulatory light chain in the LD (Fig.5) [14]. The results obtained are summarized in Fig.11. It was found that the mean amplitude of ATP-induced movement was ~6nm both around the distal end of the CD (Fig.11) and around the COD (Fig.12B), and ~3.5nm around the proximal end of the LD (Fig.11). As our experiments were made in the absence of actin filaments, we recorded the recovery stroke of individual S-1 heads, coupled with reaction, M・ATP → M・ADP・Pi , corresponding to the steps C to D in Fig.6 (Fig.12D). The S-1 head recovery stroke is believed to be the same in amplitude as, but opposite in direction to, the S-1 head power stroke (corresponding to the steps A to B in Fig.6) (Fig.12E).

We were interested in the fact that individual S-1 heads, position-marked with antibody 2, which attaches to RLR close to the COD, still exhibit ATP-induced movement, because it indicated that attachment of massive antibody (IgG) to RLR does not cause any steric clashes to inhibit S-1 movement. To solve the above puzzling result, we have examined the effect of anti-RLR antibody (=antibody 2) on both in vitro ATP-dependent sliding of actin filaments over myosin molecule fixed to a glass surface and Ca^{2+}-activated muscle fiber contraction [25]. Application of anti-RLR antibody inhibited in vitro actin filament sliding over myosin, being consistent with the result of Muhlrad et al. [23] that chemical modification of RLR inhibits in vitro actin filament sliding over myosin. Unexpectedly, however, anti-RLR antibody had no effect on muscle fiber contraction [25]. Though our research work is still in progress, the results stated above, together with our unpublished observations, can be taken to imply that (1) the swing of the CD around the COD, suggested by the swinging lever arm hypothesis may not be an active process, and may not be essential in producing filament sliding taking place in the hexagonal filament-lattice in muscle.

Much more experimental work on the role of myosin head LD and myosin S-2 is necessary for full understanding of the mechanism of muscle contraction at the molecular and submolecular levels.

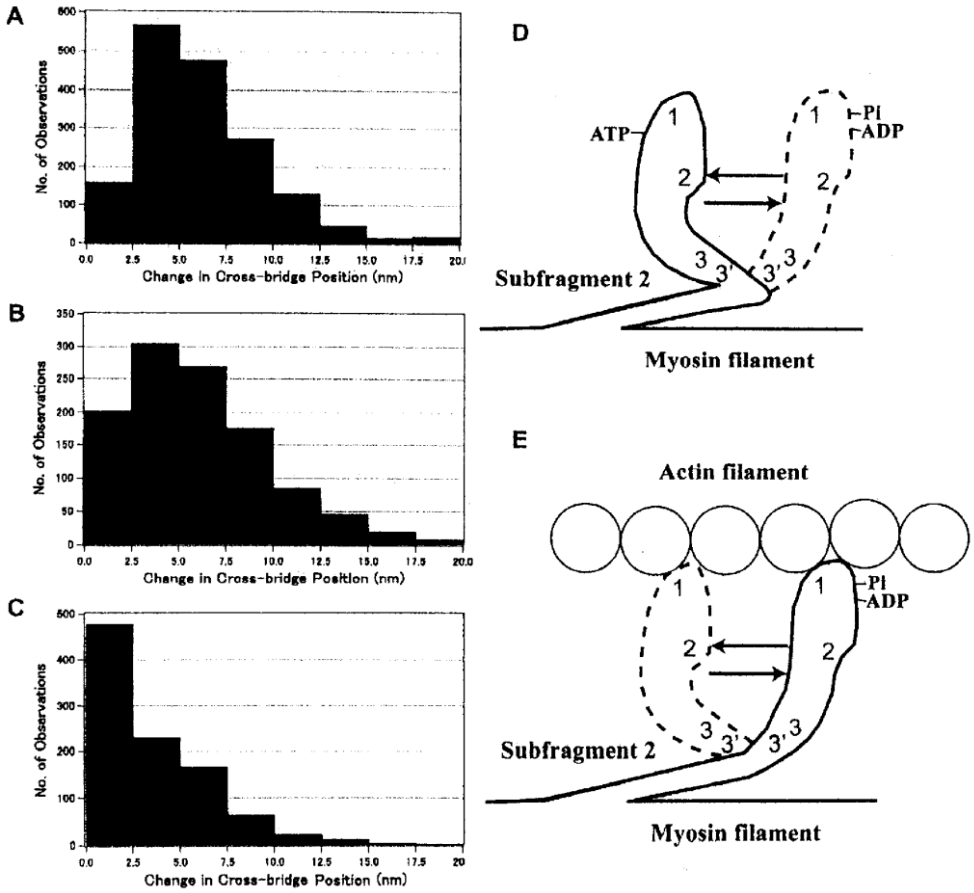

Figure 11. (A—C) Histograms of the amplitude distribution of ATP-induced myosin S-1 head movement, position-marked with antibody 1 (A), antibody 2 (=anti-RLR antibody)(B), and antibody 3 (=anti-LD antibody)(C). (D, E) Diagrams showing possible changes in shape of myosin S-1 head during the recovery stroke in the absence of actin filament (D) and during the power stroke in the presence of actin filament (E). Approximate regions of attachment of antibodies 1, 2 and 3 are indicated by numbers 1, 2 and 3, 3′, respectively [14].

4. Summary

Muscle contraction results from relative sliding between the thick (myosin) and thin (actin) filaments, which in turn is caused by the attachment-detachment cycle between the myosin heads extending from the thick filaments and the actin monomers in the thin filaments. The myosin heads (myosin subfragment 1,S-1) consists of the catalytic domain (CD) and the lever arm domain (LD), which are connected via the converter domain (COD). The S-1 is connected to the thick filament via myosin subfragment 2 (S-2). It is generally believed that each S-1 head undergoes structural changes (power stroke) while attached to actin, thus producing unitary filament sliding.

The swinging lever arm hypothesis, which assumes active rotation of the CD around the COD, is constructed on the basis of crystallographic studies on the truncate S-1, in which both the LD and the S-2 are eliminated, and therefore completely ignores possible role of the LD and the S-2. In this article, we presented evidence for the essential role of the LD and the S-2, as well as evidence against the swinging lever arm hypothesis. We emphasize that, to reach full understanding of muscle contraction mechanism, much more experimental work is necessary using experimental systems, in which connection of the S-1 to the thick filament remains intact.

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Neuromuscular Junction

Chapter 7

Non-Cholinergic Signaling Pathways at Vertebrate Neuromuscular Junctions

Artem I. Malomouzh

Additional information is available at the end of the chapter

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1. Introduction

Neuromuscular junction (NMJ) is the functional contact (synapse) between an axon of motor neuron and muscle fiber. It is generally accepted to consider this contact only as a specialized morpho-functional structure, where chemical transmission (via release of the acetylcholine (ACh)) of electrical signal from motor neuron to muscle fiber occurs, ultimately causing the muscle to contract. This synaptic contact is probably one of the most studied synapses since it has relatively large size and easy accessibility for various experimental manipulations. A great body of data is received on the development, molecular organization, morphology, and physiology of both pre- and postsynaptic regions of the NMJ. It's not so long ago that it seemed that practically all was known about the NMJ. However, due to the significant progress in the improvement and application of electrophysiological, genetic, pharmacological, biochemical and immunohistochemical methods a number of previously unknown aspects of neuron and muscle interaction were revealed. So, according to numerous studies, not only ACh (which by the way does not always lead to a contraction of the muscle fiber) is released in the vertebrate neuromuscular synapse, but also a number of other synaptically active molecules. And these molecules can be released from both nerve terminal (anterograde signal), and from muscle fiber (retrograde signal).

Before starting the consideration of the facts relating to the yet poorly studied non-cholinergic signaling, it should be recalled main points of the structure and functioning of the NMJ.

2. Neuromuscular junction organization: Brief overview

Detailed descriptions of the NMJ anatomy can be found in [1-7].

Motor neurons in the ventral region of the spinal cord send axons out toward the periphery (Fig. 1). In mammals and many higher vertebrates, each muscle fiber typically has a single

synaptic site innervated by a single motor axon branch. In front of the contact, the motor axon loses its myelin sheath and forms nerve terminal branches. Several non-myelinating Schwann cells are located over these nerve terminal branches and make processes that are closely covered to them. Terminal Schwann cells, motor nerve terminal branches and the postsynaptic specializations of sarcolemma (also known as a motor end plate) together form the neuromuscular junction (or myoneural junction).

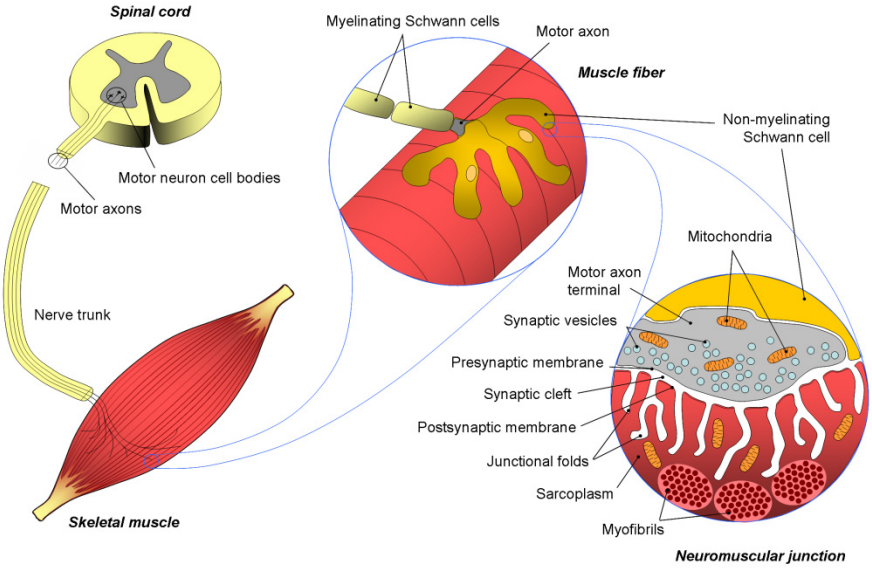

Figure 1. The anatomy of vertebrate neuromuscular junction

The motor nerve ending contains a large number of small synaptic vesicles which store molecules of the neurotransmitter ACh. The latter is synthesized in nerve terminals from choline and acetyl coenzyme A by the cytoplasmic enzyme choline acetyltransferase (ChAT) and transferred by a vesicular ACh transporter (VAChT) into synaptic vesicles. The transmitter contained in a single vesicle (in vertebrate NMJ it is about 5000 – 10000 molecules of ACh) is often referred to as a 'quantum', because during vesicle exocytosis relatively stable portion of chemical substance is released.

ACh diffuses across the synaptic cleft (50 – 100 nm) to be received by ACh receptors on the postsynaptic sarcolemma. One of the most striking structural features of this region is the deep infolding of sarcolemma. The crests of the folds contain a high density of ACh receptors whereas in the depths of the folds a density of voltage-gated sodium channels are presents. The binding of ACh to receptors causes the opening of cation-selective ion channels and allows a net flux of positive charge into the skeletal muscle. When rising depolarization is adequate to open voltage-gated sodium channels, the threshold for action potential generation is reached. Then action potential sweeps across the muscle fiber membrane and the muscle fiber contracts. The neurotransmitter action is terminated by

localized in synaptic cleft enzyme acetylcholinesterase (AChE) hydrolyzing ACh to choline and acetate. Choline is recycled into the motor nerve terminal by a high-affinity uptake system, making it available for the resynthesis of ACh.

3. Neurotransmission in neuromuscular junction

First of all it must be recalled, that ACh release from the motor nerve ending does not always leads to muscle fiber contraction, and motor neuron not only induce a contractile activity in the muscle, but also control of a number of morphological and functional properties of muscle fibers. This latter influence is usually referred to as neurotrophic and it often involve the control of gene expressions in the muscle [8-13]. At the NMJ have revealed the presence of several distinct types of ACh release: spontaneous quantal, nerve impulse evoked quantal and non-quantal release. Molecular mechanisms, features and functional significance of these secretion types are described in detail and systematized in reviews [6,14-17]. Here we will briefly consider these processes.

Spontaneous quantal release. Low amplitude (0.5 – 1 mV) potentials, called as miniature endplate potentials (mEPPs; Fig. 2A) appear in the synaptic area of sarcolemma as the result of release of a single synaptic vesicle content ('quantum'). Average mEPPs frequency in vertebrates is about 1 per second in the absence of nerve stimulation.

Nerve impulse evoked quantal release. The nerve action potential leads to opening of voltage-gated Ca^{2+} channels, resulting in the local influx of Ca^{2+} into motor nerve ending. This leads to the relatively synchronous release of 20-400 ACh quanta, what in its turn causes depolarization of the postsynaptic membrane by several – several tens of mV. This potential is recorded as an endplate potential (EPP; Fig. 2B). The number of quanta released by a single nerve impulse is known as the 'quantal content' of the EPP.

Non-quantal release. In the absence of nerve stimulation the amount of ACh released by non-vesicular manner is a hundred times greater than ACh, released by spontaneous quantal release. Electrophysiologically, the intensity of the non-quantal ACh secretion process can be evaluated only in terms of AChE inhibition [17; Fig. 2C]. Currently there is still no clear answer to the question of which protein is mediated by this type of neurotransmitter release. Nevertheless it is established that this process is not a passive leakage, and it is regulated by various synaptic active molecules, regardless of quantal release processes [17] and is blocked by increasing of Mg^{2+} concentrations [18], as well as inhibitors of both vesicular ACh transport (vesamicol) [19,20] and choline uptake (hemicholinium-3) [21].

Although molecular mechanisms of action of spontaneously released mediator are not yet fully established, the majority of data indicate that tonic neurotransmitter release is one of the neurotrophic control factors whereas the physiological role of the evoked quantal ACh release is to ensure clear transmission of the electric impulse from the motor nerve to the muscle fiber [15,17]. At the same time obtained experimental results suggest a possible trophic role of ACh released by quantal manner in response to the nerve action potential [8,22]. However, until recent time, the fact that other signaling molecules can be released

together with the ACh from motor nerve endings and participate in the neuromuscular transmission was ignored.

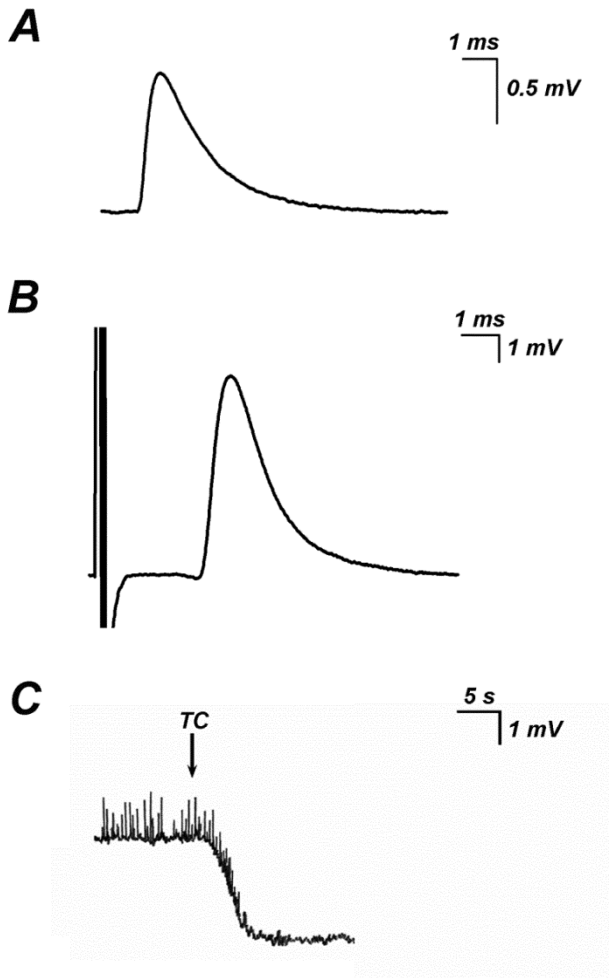

Figure 2. Electrophysiological registration of different types of ACh release at the rat neuromuscular junction. **A** – Spontaneous miniature endplate potential (averaging of about 100 signals in a single fiber) as a result of the action of individual quantum of ACh molecules. **B** – Evoked endplate potential (averaging of about 100 signals in a single fiber) as a result of synchronous release of a number of quanta of neurotransmitter in response to a nerve impulse. In this case, the phasic ACh release was reduced by high Mg^{2+} in the Ringer's solution to prevent muscle contraction. **C** – Endplate hyperpolarization (H-effect) following blockade of skeletal muscle postsynaptic nicotinic receptors by (+)-tubocurarine (TC) and cholinesterase as a result of the action of ACh, released predominantly in a non-quantal manner.

4. Cotransmission and neuromodulation

In neurobiology for decades the 'Dale's principle' dominated, according to which, one neuron synthesizes, stores, and releases a single transmitter liberated from all own's axon terminals. In this regard, vertebrate motoneuron for a long time considered as a cell capable to release ACh only. However by the early 90's a large amount of experimental data was obtained, the analysis of which led to the formation of the modern theory of 'cotransmission' [23-28]. According to this theory, one or several types of synaptically active molecules – cotransmitters (coexisting transmitters) are released from the neuron together with basic mediator. These cotransmitters are capable of exerting its own effects in the target cell, regulating the release of primary neurotransmitter (presynaptic modulation) or modulating the physiological response in the postsynaptic cell (postsynaptic modulation). At present, it can be stated that the phenomenon of corelease of several neurotransmitters from the nerve endings is the rule rather than the exception for the entire nervous system, including peripheral part [24,25,27,28].

Some signaling molecules that do not meet the definition of 'cotransmitters' are involved in the functioning of the synaptic apparatus too. They are released from either neuron, but independently of the primary neurotransmitter, or have a glial origin or they are released from the postsynaptic cell and, along with cotransmitters, exert their modulating and/or neurotrophic effects.

5. Purinergic signaling

Purines in NMJ. Adenosine 5′-triphosphate (ATP) one of the purine compounds, which plays a crucial role in energy exchange and metabolism in all living cells. It is formed from adenosine 5′-diphosphate (ADP) and inorganic phosphate by the enzyme ATP synthase, localized in mitochondria. These organelles are abundantly represented in all synapses, including NMJ, where they concentrated both presynaptic and postsynaptic regions. The ATP concentration is about 2-5 mM in the cytoplasm of most neurons, whereas directly in the synaptic vesicles it is higher for at least by 2 orders, including vesicles of cholinergic terminals [29,30], where it is pumped by ADP/ATP translocase [31,32]. It is necessary to note that in ACh contained vesicles besides ATP, small amounts of ADP and traces of adenosine 5′-monophosphate (AMP) were observed [29,30].

Release and metabolism of purines in the synaptic NMJ cleft. In experiments on rat and frog neuromuscular preparations clear evidence of Ca^{2+}-dependent corelease of ATP and ACh from motor nerve terminals was obtained [33-35]. At the same time, there are data suggested that ATP is released from skeletal muscle cells in response to muscle contraction [35-37]. And if in the first case, mechanism of a signaling molecule release is exocytosis of synaptic vesicle, in the case of the muscle fiber molecular mechanism of ATP discharge is not yet fully established. At the present time pannexin (Pnx) hemichannels have also been proposed as relevant ATP conduits [38]. In any case, nowadays the fact of a significant increase of the ATP concentration and its derivatives in the synaptic cleft of the NMJ after motor nerve stimulation is well established [29,39].

Like many signaling molecules, ATP released from the cell is metabolized in the extracellular space. ATP is broken down to ADP and AMP by extracellular ATPases [29]. Further, as shown directly in the rat NMJ, AMP was either dephosphorylated into adenosine by ecto-5′-nucleotidase or deaminated into inosine monophosphate by ecto-AMP deaminase [39]. Inosine is an inactive metabolite [40], but adenosine is a signaling molecule that activates its own receptors [41]. Formed adenosine is removed from the synaptic cleft of the NMJ by dipyridamole-sensitive adenosine uptake system, and there are reasons to believe that adenosine uptake is more important than adenosine deamination in the regulation of extracellular adenosine concentrations [40].

Purine receptors in NMJ. Purine receptors are divided into 2 large groups: adenosine or P1 receptors and P2 receptors, which activated by nucleotides ATP, ADP, uridine 5′-diphosphate and uridine 5′-triphosphate [29,41-43]. All P1 receptors (A_1, A_{2A}, A_{2B} and A_3) are G protein-coupled receptors, while P2 receptors are consist of two distinct families: P2X receptors, which are ligand-gated ion channels for cations, and P2Y receptors, which are G protein-coupled receptors. Seven mammalian P2X receptor subtypes ($P2X_{1\text{-}7}$) and eight mammalian P2Y receptor subtypes have yet been cloned and functionally defined as P2 receptors ($P2Y_{1,2,4,6,11,12,13,14}$) [29,41].

Pharmacological evidence of the presynaptic localization of adenosine (P1) receptors were obtained on preparations of NMJ, both in amphibians [44] and mammals [45,46]. In the latter case the presence of A_1 and A_{2A} receptor subtypes on the nerve ending was defined. Subsequently, confirmation of exclusively presynaptic localization of A_{2A} receptors in the the NMJ of mouse was obtained by the means of immunohistochemistry [47]. At the same time, on the plasma membrane of human skeletal fiber adenosine A_{2A} and A_{2B} receptors were revealed by means of immunohistochemistry [48].

As for P2 receptors, the following is known at present time. $P2X_7$ receptor subunits were found on presynaptic motor nerve terminals of mouse, but there is no evidence for $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$ or $P2X_6$ receptor subunits [49]. According to a number of electrophysiological studies metabotropic P2Y receptors are also localized on the motor nerve endings of both amphibian and mammals NMJ [44,50-52]. However, P2Y receptors were found on the postsynaptic membrane of skeletal muscle fiber. Moreover, the presence of $P2Y_1$ and $P2Y_2$ receptors on the plasma membrane was precisely established [53,54]. Developing mammalian skeletal muscle fibers are able to express 4 subtypes of metabotropic purine receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_{11}$) and, what is interesting, all types of P2X receptors which, apparently, are absent on the mature innervated muscle fibers [29,55-57].

The physiological significance of purinergic signaling in NMJ. Quite a lot of evidence indicating the significant role of purinergic signaling in the processes of establishment, development and maintenance of NMJ is accumulated. In developing of the *Xenopus* neuromuscular synapses ATP increased the intensity of the ACh release from the nerve ending and enhanced the responses of the muscle membrane to ACh [58,59]. Moreover, ATP modulates agrin-induced ACh receptor aggregation via activation of $P2Y_1$ receptors [60] and regulates

the expression of AChE and ACh receptor genes via activation of $P2Y_2$ receptors [53]. Activation of ionotropic ($P2X_4$, $P2X_5$ and $P2X_7$) and metabotropic $P2Y_1$ and $P2Y_4$ purinoreceptors participates in forming the calcium transients of multinucleated myotubes [56]. The significance of purinergic signaling in the development of neuromuscular synapses is perfectly demonstrated in the work [57], where authors investigated the NMJ in knockout mice ($P2X_2$). It should be recalled that this type of receptor is absent on mature muscle fiber, where it is expressed only in the early stages of development. It was found that the neuromuscular contacts in these knockout animals have significant structural abnormalities, followed by muscle fiber atrophy.

In addition to the role of ATP in the process of synaptogenesis a lot of data is obtained about the modulator effects of purines on the processes of ACh release in the mature vertebrate NMJ. So, it was found that ATP and adenosine significantly reduced the intensity of both evoked and spontaneous quantal release of ACh, activating presynaptic purine receptors [44,50,51,61,62]. However adenosine can also facilitate the quantal release of ACh what, apparently, depends on the pattern of motor nerve stimulation [46]. Extracellular ATP induces presynaptic inhibition of ACh release via its own P2Y receptors, which modulate voltage-gated Ca^{2+} channels [50,51]. Adenosine also inhibits quantal release of ACh, acting through P1 receptors and its mechanism of action does not affect the operation of calcium channels [50,51]. As for the influence of purines on the non-quantal release of ACh it is established that its intensity remains unchanged in the presence of adenosine, but it decreases via activation of P2Y receptors by the ATP molecules and this mechanism is not coupled to presynaptic voltage-dependent Ca^{2+} channels [52,63].

Postsynaptic modulator effects of purines in the mature neuromuscular synapse were also established. So it was found that ATP can increase ACh receptor activity [64-66] and inhibit chloride channels in mammalian skeletal muscle [54].

6. Glutamatergic signaling

The origin and localization of glutamate in NMJ. Glutamate and its derivatives are dominant in terms of numbers among all amino acids in nervous tissue. This amino acid plays not only a central metabolic role [67,68], but also acts as the primary excitatory neurotransmitter in the central nervous system [69-71].

In experiments on the culture of spinal neurons and skeletal muscle fibers of *Xenopus* embryos it was shown that glutamate is present in the growth cone of developing motor neurons and in the nerve endings forming synaptic contact with muscle fiber [72]. Significant immunoreactivity to glutamate has been identified directly in the nerve endings of mammals [73,74], and the level of immunoreactivity in terminals that innervate the extensor digitorum longus was higher than in the nerve endings of soleus muscle. Concentration of glutamate in the motor nerve endings of the extensor digitorum longus was estimated in the range 10-20 mM [74]. Furthermore, authors were able to demonstrate

direct association of glutamate with synaptic vesicles what supposes the joint release of ACh and glutamate in the synaptic cleft [74].

Glutamate release from the motor neuron. The uptake of labeled glutamate by frog motor neurons and its release from the motor nerve terminals were demonstrated in one of the first studies indicating the possible involvement of glutamate in the functioning of the vertebrate NMJ [75]. The detection of the vesicular glutamate transporters (VGLUT1 and VGLUT2) in the motor neurons of the spinal cord testifies for vesicular release of amino acid from cholinergic terminals [76]. The VGLUT3 transporter was found directly in the motor nerve terminals [77]. Clear evidence for co-operative glutamate release with ACh was obtained in the study of synaptosomes from nerve terminals of the *Torpedo* electric organ [78]. Previously this object considered as 'purely cholinergic system' and used as a classical model for studying the general aspects of the cholinergic neurotransmission. The authors have shown the corelease of these two mediators in Ca^{2+}-dependent manner under the action of depolarizing agents [78]. This fact of a simultaneous release ACh and amino acid was confirmed later by other authors [79]. Electrophysiological data showing action potential induced corelease of glutamate and ACh from of mammals motor neurons was obtained recently [80,81].

Glutamate receptors in the NMJ. Nowadays this is probably the most studied aspect of glutamatergic signaling in the neuromuscular synapse. A wide range of ionotropic glutamate receptors (kainate, AMPA and NMDA) has been found in synaptic contact in experiments on the culture of neurons and myocytes of *Xenopus* [72,82,83], and data indicate about predominantly presynaptic localization of these receptor structures. In the later stages of amphibians development, namely in tadpoles and adult frogs metabotropic glutamate receptors were found [80,84-86], which, apparently, are localized postsynaptically [80,85]. In contrast to the amphibian NMJ, in the endplate of mammals to date were found only ionotropic glutamate NMDA and AMPA receptors, and all the experimental data show exclusively postsynaptic localization of these proteins [87-92].

Removal of glutamate from the synaptic cleft. Any extracellular enzyme which inactivates glutamate in extracellular space is not found until now [67]. It is interesting to note that the uptake of glutamate molecules from the solution, where muscle was incubated, and the transport of amino acid on sciatic nerve to the spinal cord was demonstrated even in 1967 [75]. Currently, five membrane transporters of this amino acid are identified and only two of them are widespread: GLAST (EAAT1) and GLT (EAAT2). The presence of glutamate transporter GLAST, which is localized mainly on the presynaptic membrane of Schwann cell, was revealed in experiments on the frog nerve-muscle preparation [80]. GLAST and GLT transporters were found in mammals in the area of endplate and they are fairly well represented in the synapses of both fast and slow muscles [93]. And it was established that most of them are localized deep in postsynaptic folds [93].

The physiological significance of glutamatergic signaling in the NMJ. It is established that glutamate affects the processes of ACh release from motor nerve endings, and the mechanism of modulator action, based on available data, in mammals and amphibians is different. Moreover, in amphibian it, apparently, changes during ontogenesis. Thus, it is

shown that glutamate facilitates the quantal release of ACh at early stages of establishment and maturation of the NMJ in frog [72,82,94], whereas in adult animals, on the contrary, the amino acid inhibits the quantal release of ACh [80,84-86]. At the same time any effect of glutamate on the quantal release of ACh in the NMJ of mammals was not established [89], however, the inhibition of non-quantal ACh release was revealed [89,90]. And since this type of the mediator is able to perform trophic function [17], in this case, glutamate may be considered as a regulator of neurotrophic control of the properties of the postsynaptic membrane. Due to the fact that the activation of glutamate receptors both in amphibians [85], and mammals [88-90] may be accompanied with increased synthesis of nitric oxide molecules (NO), then it should be assumed that the amino acid is able to participate in a wide range of physiological functions, since the contribution of NO-mediated signaling was revealed in metabolism and contraction of muscle fibers [95,96].

7. Peptidergic signaling

N-Acetylaspartylglutamate (NAAG) is the most abundant and widely distributed neuropeptide in the mammalian central nervous system, able to perform signaling function in the interneuronal synapses [97].

High concentrations of NAAG have been found in spinal cord motoneurons and motor components of cranial nerve nuclei [98-100]. Moreover, this dipeptide was found in sciatic nerve [98,101] and phrenic nerve terminals [87]. NAAG can be involved in neurotransmission as: (i) direct agonist of glutamate ionotropic NMDA receptors and metabotropic GluR3 receptors and (ii) as a glutamate precursor, which is formed directly in the extracellular space during hydrolysis by the enzyme glutamate carboxypeptidase II (GCP II), also known as N-acetylated α-linked acidic dipeptidase (NAALADase) [102].This peptidase is a membrane-bound protein which was detected in non-myelinating presynaptic Schwann cells surrounding motor nerve terminals [87,103].

Experiments on rat NMJ showed that NAAG is able to depress non-quantal ACh release [90]. The mechanism of neuropeptide action is realized through its extracellular hydrolysis by the GCP II with the formation of glutamate molecules, which, as was shown earlier [89], activate glutamate postsynaptic NMDA receptors and thereby trigger the NO-mediated mechanism of reducing the intensity of the non-quantal ACh release [104].

Substance P. This peptide belongs to the tachykinin neuropeptide family, found in neurons of both central and peripheral nervous system, where it performs neurotransmitter and neuromodulator functions [105]. The main receptor for substance P is G protein-coupled neurokinin 1 (NK-1) receptor.

The presence of substance P in frog motor nerve endings was shown by immunohistochemistry [106]. Later, data demonstrating the neuropeptide release during the stimulation of the motor nerve was obtained [107]. NK-1 receptors, localized in perisynaptic Schwann cells NMJ were found by the same authors. Substance P was not found by

immunohistochemistry in the motor nerve endings of rodents [108,109], however, it was found in the muscle fibers. Soleus muscle had a significantly higher content (0.61 ng/g) than the extensor digitorum longus (0.22 ng/g) [109].

In studying the signaling function of substance P in the frog NMJ its influence on all compartments NMJ was revealed: on motor nerve terminal, on postsynaptic membrane and on Schwann cell. So, following effects were shown: (i) facilitating effect of neuropeptide (at a concentration till 1 μM) on spontaneous and evoked quantal release of ACh [110]; (ii) reduction of the sensitivity of the postsynaptic membrane to ACh at the concentration peptide above than 1 μM [111,112]; and (iii) induction of Ca^{2+} release from internal stores in Schwann cells [107]. In the mammalian NMJ also was noted presynaptic facilitatory action of substance P. Neuropeptide facilitated the indirect twitch responses of the rat diaphragm and increased amount of ACh released into the bathing medium in response to tetanic stimulation of the phrenic nerve [113].

Calcitonin gene-related peptide (CGRP). This peptide is distributed throughout the central and peripheral nervous systems and exhibits a range of biological effects [114]. CGRP mediates its effects via G protein-coupled receptor called calcitonin receptor-like receptor (CALCRL).

Frog motor neurons express CGRP-like immunoreactivity and this immunoreactivity in motor nerve terminals is confined within so called 'large dense-core vesicles' [115]. CGRP-like immunoreactivity was found in the mouse and rat motor nerve terminals [116,117]. In rat hind limb CGRP-like immunoreactivity is heterogeneously present in the endplates and, apparently, correlates with the muscle fibers phenotype [118]. Motoneurons of small and slow-twitch motor units in general have lower levels than motoneurons of large and fast-twitch motor units [119]. It is established that the CGRP is released by nerve impulse activity [120]. Calcrl mRNA and CALCRL protein were found directly in postsynaptic region of rats muscle fibers [121]. The CGRP receptor, and its two associated components (RAMP1 and RCP), are highly concentrated at the adult avian NMJ where they co-localize with AChE and ACh receptors [122].

Physiological role of CGRP was revealed not only at establishment and development NMJ, but also in the process of its functioning. Thus, on cultured chick myotubes it was shown that the CGRP stimulates the turnover of phosphoinositides and the accumulation of inositol phosphates [123] and also increases the number of surface ACh receptors [124]. In 1-day-old *Xenopus* nerve-muscle cultures CGRP enhances the postsynaptic response at developing NMJs by increasing the burst duration of embryonic ACh channels [125]. Moreover, neuropeptide plays a key role in the trophic regulation of AChE at the NMJ not only during synaptogenesis, but lifelong [122,126].

In experiments on mature rodent neuromuscular synapse it was shown that CGRP enhances muscle contraction during stimulation of the nerve fibers or direct stimulation of the muscle [117]. The ability of neuropeptide to enhance the intensity of spontaneous quantal ACh release was revealed [127]. The effect of the CGRP facilitating the secretion of ACh was also described in the frog neuromuscular synapse [128].

8. Nitric oxide signaling

Biosynthesis of NO in NMJ. NO is a free radical short-living (half-life 4-6 s) gas, which is formed from L-arginine in the body by the enzyme NO-synthase. Three isoforms of this enzyme were identified: neuronal (type I), inducible (type II) and endothelial (type III) [96,129]. Health skeletal muscular tissue expresses both endothelial and neuronal isoforms of the NO-synthase [96]. Endothelial isoform is co-localized with mitochondria of skeletal muscle fibers [130], while the neuronal NO-synthase is concentrated in the NMJ [88,131,132]. 'Anchoring' of the enzyme in the postsynaptic membrane is provided by interaction with the dystrophin-associated protein α1-syntrophin [131]. Moreover, experiments on skeletal myotubes showed that neuronal NO-synthase is able to interact directly with the NMDA receptor via the PSD-95 protein [133]. In mature muscle of health rodents and human the expression of inducible NO-synthase is absent or represented very poorly [134,135], however, it can significantly increased under certain pathological conditions [135,136].

Neuronal and endothelial NO-synthases are activated by calcium and calmodulin, whereas the inducible isoform binds irreversibly to the calmodulin right after the translation, so this enzyme produces NO independently of changes in intracellular calcium concentration [96]. It is established that during muscle contraction the activity of NO-synthases increases by several times [137,138]. It is well explained by the increase of cytosolic calcium concentration, which facilitates the interaction of the enzyme with calmodulin. According to several authors skeletal muscle produces from 2 to 25 (average ~ 10) pmol $\cdot$ min^{-1} $\cdot$ mg^{-1} of nitric oxide [137,139,140].

It is interesting to note that, apparently, in amphibians the localization of NO-synthases is differ from mammals. So, in frog NMJ NO-synthase immunostaining was found at the membrane and occasionally in the cytoplasm of perisynaptic Schwann cells and was not detected in the nerve terminal or muscle [141].

Physiological effects of NO in NMJ. The mechanism of NO signaling function is based on its interaction with thiol groups and/or transition metals in proteins. Most of the NO physiological responses are mediated by S-nitrosylation of redox centers and interactions with heme or nonheme iron and copper. Thus, the binding of NO with heme-containing protein leads to changes in the activity of the latter: in the case of cytochrome-c oxidase - inhibition and in case of guanylate cyclase - activation [86].

NO-mediated signaling plays a certain role in the formation of the NMJ. In particular, the role of NO both in presynaptic and postsynaptic differentiation of NMJ was shown [142,143]. In mature neuromuscular synapse physiological significance of NO-mediated signaling was revealed in processes metabolism and contraction of muscle fiber, as well as in modulation of ACh release from the motor nerve ending.

It is shown that the NO-synthase activity can modulate mitochondrial respiration in skeletal muscle. So, inhibitory effect of NO on oxygen consumption of muscle tissue was revealed [144,130]. Modulatory influence of NO was demonstrated with respect to carbohydrate metabolism. It was shown that NO-synthases blocking inhibits the reuptake of 2-

deoxyglucose, whereas exogenous NO molecules donor leads to its increase [138,139]. On the other hand, the possibility of NO to inhibit the activity of glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase was revealed [145]. Finally, data on the NO-mediated inhibition of the creatine kinase activity in skeletal muscle was obtained [146]. It can lead to decrease in the synthesis of ATP from creatine phosphate.

The action of NO on contractile function of muscle fibers is complex. *L. Kobzik* and co-workers [137] showed that NO-synthase inhibition, inactivation of extracellular NO and inhibition of guanylate cyclase increase the amplitude of muscle contractions, which is reduced in the presence of NO donor and with an increase the cGMP concentration. At the same time *R. Morrison* and co-workers [147] demonstrated reduction of the maximum rate of contraction of muscle fibers on rat diaphragm during NO-synthase inhibition. This did not happen, when the donor of NO was added together with the blocker of enzyme. Similar results were obtained also on mouse extensor digitorum longus [148]. Interesting data is presented in [149], where it is shown that L-arginine (the substrate for the NO synthesis) increases the amplitude of muscle contraction in response to nerve stimulation of isolated rat diaphragm, but it leads to a reduction at direct muscles stimulation. Both effects removed by NO-synthase inhibition and were not appeared after D-arginine application. The authors suggest that NO enhances contractile function by acting on presynaptic level, and reduces it when acting on postsynaptic. One of the major potential targets for NO on postsynaptic compartment is the ryanodine receptors of sarcoplasmic reticulum. NO can both facilitate and inhibit the activity of the ryanodine receptors [150,151] what, apparently, explained by the presence of several areas in this protein, interacting with the NO molecules [96].

Namely the fact of finding the post-synaptic localization of NO-synthase and modulating effect of NO molecules on the process of ACh release from motor nerve terminals allow us to declare that this signaling molecule acts as a retrograde synaptic mediator in the NMJ. NO reduces the intensity of both spontaneous and evoked quantal ACh release in the neuromuscular synapse of the frog [152,153]. The inhibitory action of nitric oxide on spontaneous and induced synaptic currents was shown also in the developing neuromuscular contacts *Xenopus laevis* [154]. In contrast with endplate of amphibian, NO has no effect on spontaneous and evoked forms of quantal ACh release, but significantly reduces the intensity of non-quantal release of ACh in mammalian NMJ [104]. However, as was shown later, NO can modulate the quantal release in mammals, enhancing transmitter release from motor nerve via a cGMP pathway, but it occurs only when adenosine A_1 receptors were blocked [155]. It is also necessary to note the fact that in the synapse of amphibians and mammals, the fact endogenous tonic effect of NO on ACh release processes was confirmed repeatedly [86,89,104,153].

9. Conclusion

Until now, many people share the opinion that intercellular contact between motor neuron and skeletal muscle fiber is very well studied morpho-functional structure, which provide the one-way transmission of electrical impulse from the motor neuron to the muscle for the

initiation of the contractile act. However, this opinion is totally wrong and one of the proofs for that is this review which describes a number of most studied signaling pathways mediated by molecules that previously were not considered in the aspect of the functioning of the NMJ. Experimental facts proving; (i) the formation of these molecules in the neuromuscular synapse; (ii) their release in the synaptic cleft; (iii) the interaction with specific receptor proteins; and (iv) the existence of a specific physiological effect for each of these signaling molecules are presented and analyzed here. It is necessary to emphasize that the author intentionally considered those signaling molecules (ATP, glutamate, NAAG, substance P and NO), which act as an individual neurotransmitter in the mature organism, but in synapses of other parts of the nervous system [27,29,69,105,156,157]. CGRP, in its turn, also plays its role in mature intercellular contact, acting as a cotransmitter in sensory-motor neurons [27].

A number of signaling molecules which are also participate in the signaling between motor neuron, Schwann cell and skeletal muscle fiber remained beyond the review. At least nerve growth factor (NGF), glial-cell-line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4) and transforming growth factor-beta 2 (TGF-β2) are among them. The main role in regulating of neuronal survival, plasticity, growth, and death is ascribed to them. However, it turned out that these proteins act also as regulators of the maintenance, function, and regeneration of skeletal muscle fibers [158]. So, it was shown that BDNF, NT-3, NT-4 are expressed both in motor neurons and in muscle fibers. GDNF, in its turn, is expressed in Schwann cell and in muscle fiber. Activity-dependent synthesis and release of these factors in extracellular space have been reported. Receptors for all these factors were revealed in mature NMJ, their participation in the regulation of neuromuscular transmission was shown also at the expense of influence on the processes of ACh release [158-160].

Thus, NMJ is a rather complicated and flexible compartment for multicircuit intercellular communication between a motor neuron and muscle fiber, what provides the synaptic plasticity and reliability of synaptic transmission.

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10. References

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Use and Disuse

Chapter 8

Prevention of Skeletal Muscle Wasting: Disuse Atrophy and Sarcopenia

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Additional information is available at the end of the chapter

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1. Introduction

Skeletal muscle plays a considerable role in health and disease. Muscle mass is essential for health and survival and plays a major role in mobility as well as morbidity and mortality. There is continual synthesis and degradation of proteins as part of normal metabolism and homeostasis. Equally remarkable, is the characteristic of plasticity allowing muscle to change and adapt depending on the stimuli and load placed upon it.

Increasing the contractile load on skeletal muscle leads to increased muscle mass and strength typified by that seen with a resistance exercise programme. This can be beneficial for sports performance as well as allowing an individual to improve physical fitness, maintain health status and improve quality of life.

Inactivity, or reduced load, results in a loss of skeletal muscle mass. This loss of muscle mass and corresponding loss of strength and function may lead to a reduced quality of life and life expectancy [1].

The morphological changes associated with muscle atrophy are a decreased cross-sectional area (CSA) of muscle fibres resulting in a reduced muscle mass, but without a decrease in the number of muscle fibres. When the muscle becomes smaller, this leads to reduced muscle strength. In humans, muscle atrophy appears to be a consequence of reduced protein synthesis with no change in protein degradation [2]. Loss of muscle mass is also seen in disease states such as cancer, AIDS, renal failure, congestive heart failure, chronic obstructive pulmonary disease (COPD) and burns. In addition to disuse, disease-induced muscle loss (cachexia) also involves a complex interplay of cytokine and inflammatory responses.

Disuse is a broad descriptor of the mechanical unloading of the muscle; with the most extreme example being that of spaceflight. Far more common life events, such as immobilisation, bed

rest or disuse also include decreased mechanical loading. Perhaps most common is the lack of regular mechanical loading that accompanies sedentary behaviour. Changes seen with disuse/inactivity do not include an increase in inflammatory or cytokine response such as that seen in disease state cachexia.

In this book chapter we will discuss various conditions which lead to skeletal muscle atrophy. The excitement generated by spaceflight led many researchers to study physiological effects influencing the astronaut who has to function in an atmosphere of no gravity. The most common simulation of spaceflight is ground based bed rest. While an individual is placed in the horizontal position, the changes noted are not completely identical to spaceflight since the astronaut conducts normal daily tasks while the individual on bed rest must restrict their movements. Physiological changes during bed rest include decreases in muscle mass and strength and other disuse associated changes in skeletal muscle. If the bed rest is at a 6^{o} head down tilt angle, the individual will experience equivalent fluid shifts to that seen in spaceflight.

A number of immobilisation techniques have been identified to investigate disuse and load reduction on skeletal muscle. Unilateral lower limb suspension (ULLS) involves one limb being suspended while the other is used for movement assisted by crutches. Since the suspended leg cannot move, muscle mass loss is observed. Similarly, a limb immobilised by a leg brace or cast also loses muscle mass due to disuse.

A rodent model of disuse, hindlimb suspension, also leads to significant skeletal muscle atrophy. The model involves the animals supporting their weight on their front legs and preventing the back legs from bearing any weight because the animal is suspended by the tail. The unloading leads to muscle atrophy and provides a crucial animal model of muscle atrophy to provide insight into the complex interplay of influences on skeletal muscle with inactivity.

The extent of muscle atrophy induced by these models has been reported by Narici and de Boer [3] and a brief summary is provided here:

i. Spaceflight: reductions of total lower limb muscle mass of between 6-24%.
ii. Animal models of disuse: preferential decreases in muscle fibre size are noted in slow twitch muscles which are usually consistently more activated due to their postural function.
iii. Unilateral lower limb suspension (ULLS) in humans: 5-10% decrease in quadriceps CSA within 4 weeks [4].
iv. Immobilization (cast or leg brace) in humans: 12% decrease in leg volume with 46% decrease in type I fibre size and 37% decrease in type II fibres [5].

One of the consequences of a shift in fibre type from slower phenotypes (more oxidative) to faster phenotypes (more glycolytic) is the greater fatiguability of the faster fibres. Other changes which occur during inactivity and disuse include metabolic alterations including reduced insulin sensitivity [6], decreased capillary density in both fibre types and a disruption of the skeletal muscle architecture [7].

Sarcopenia is the loss of muscle size and strength in the ageing process. There is a gradual decrease in physical function throughout the ageing process. Loss of muscle mass in the elderly is a significant health risk leading to impairment of maximal aerobic capacity, decreased insulin sensitivity, impaired oxidative defense, lower resting metabolic rate and functional dependency (reviewed by [8,9]). The mechanisms causing sarcopenia are different to those of disuse atrophy even though the reduced activity noted in most elderly individuals does contribute to skeletal muscle loss. There are a number of factors which contribute to the age-associated decrease in size and strength of skeletal muscle, including excessive oxidative stress, degeneration of the neuromuscular junction, fibre denervation/reinnervation, decreased oxidative capacity, hypoplasia of type II fibres, declining hormone levels, and a fast-to-slow fibre type transition.

Skeletal muscle changes associated with ageing begin in the 20s and decline more rapidly as a person reaches their 50s [10] and this is particularly noted in the lower body [11]. Type II fibre size may be reduced by 20-50% and although type I fibres are less affected, they may still be 1-25% decreased in size. The reduction in whole muscle mass is greater than muscle fibre size reduction because of an additional loss of fibres [10,12]. This phenomenon, not seen in disuse atrophy, is a result of progressive degeneration and reinnervation of alpha motorneurons. Denervation leads to loss of fibre number (hypoplasia) contributing to muscle atrophy. With reinnervation, the previously denervated fibres undergo a change in phenotype to the new innervation which results in an increase in number of type I fibres. This specific reduction in numbers of type II fibres is associated with increased co-expression of myosin heavy chain isoforms and fibre grouping ultimately leading to a change in recruitment as well as decreased strength.

Ageing also leads to decreased aerobic capacity, with contributory factors including decreases in stroke volume, heart rate and arterio-venous oxygen difference [13]. Together, the reduced aerobic capacity and loss of skeletal muscle mass contribute to increased morbidity and mortality [14]. Other conditions, also associated with ageing, contribute to sarcopenia, loss of function, morbidity and mortality.

Older adults often have an increased fat mass (sarcopenic obesity) which contributes to various metabolic disorders including diabetes, obesity, and cardiovascular disease; and loss of bone which causes further mobility reductions because of osteoporosis [15]. The increase of fat and non-contractile tissues in the muscle, as well as insulin resistance and reduced muscle metabolism, causes the quality of the remaining muscle in an ageing individual to be reduced [16].

There are many contributing factors to the ageing process which involve hormonal, nutritional, immunological and neural components which create a decreased pro-anabolic environment and an increased catabolic environment. For example, an increased production of cytokines and resulting inflammation. These factors contribute to sarcopenia and result in a further reduction in physical activity, decreased basal metabolic rate (BMR), increased risk of osteoporosis and increased incidence of falls and injury.

An increase in reactive oxygen species (ROS) over the lifespan also has a significant impact on the ageing process. As the body ages, an increased production of ROS as well as a reduced ability to quench the ROS molecules leads to cellular damage. Further to this detriment, there is a decrease in mitochondrial volume as well as reduced functional ability of the remaining mitochondria. This, in turn, leads to further increases in ROS production. Whereas young muscle is capable of adapting by synthesizing additional enzymes to neutralize oxidative stress, this does not occur sufficiently in older adults, thus there is accumulation of oxidative damage in the cell contributing to further mitochondrial dysfunction and malfunction of other proteins damaged by the free radicals [17].

Muscle cells are the most protein dense of all cells and there are mechanisms in place aimed at protecting and regenerating healthy muscle tissue. During atrophy, these cellular and molecular mechanisms are not able to balance the mechanisms inducing muscle loss.

The next section of this book chapter aims to elucidate:

- cellular mechanisms involved in disuse atrophy and sarcopenia including satellite cells, and myonuclear domain size.
- the most relevant molecular pathways: Myostatin/SMAD pathway; ubiquitin proteasome pathway and the IGF1/mTOR pathway.

Thereafter, the rest of the chapter will:

- Discuss methodological analysis and imaging of skeletal muscle changes with atrophy and sarcopenia (including human and animal models).
- Highlight current knowledge of ways to prevent or alleviate muscle atrophy and sarcopenia including exercise and nutritional interventions.

1.1. Myonuclear Domain and Atrophy/Sarcopenia

Skeletal muscle cells are large and one of the few cells in the body which are multinucleated. Each myonucleus governs a surrounding area of cytoplasm, called a myonuclear domain; and muscle fibres are considered to be mosaics of these overlapping myonuclear domains [18]. The myonuclear domain theory suggests that each myonucleus produces enough protein to support a limited amount of cytoplasm and the structural proteins produced are restricted to that domain [19]. If there is need for substantially more proteins, more myonuclei are required, thereby establishing new myonuclear domains as fibres increase in size.

Myonuclei are post-mitotic, but satellite cells can proliferate when required and provide new myonuclei to skeletal muscle fibres. Satellite cells are skeletal muscle stem cells which reside between the basal lamina and sarcolemma of skeletal muscle fibres. They are required for post-natal growth of the fibre [20]. When activated, satellite cells proliferate expressing the early myogenic regulatory factors (MRFs), MyoD and Myf5; thereafter they express the later MRFs, Myogenin and MRF4, differentiate and donate daughter nuclei to the muscle fibre. Satellite cells become activated when adult muscle fibres are stimulated to undergo hypertrophy or regeneration [21].

The muscle fibre maintains a relatively constant myonuclear domain size during growth [22]. Increases in CSA are often accompanied by increases in myonuclei [23,24]. Based on this theory, with muscle atrophy there should be a decrease in myonuclear number which should correspond with a decrease in CSA, in order to maintain myonuclear domain size. Indeed, atrophy due to microgravity is associated with decreased myonuclear number and a constant myonuclear domain size [7]. However, recent studies of single fibres suggest that nuclear domain may be less consistent than previously thought and it has been suggested that fibres undergoing atrophy do not lose myonuclei [25]. After 28 days of denervation, nerve impulse block or mechanical unloading there was significant muscle fibre atrophy but no loss of myonuclei [26].

Results on myonuclear domain maintenance in ageing muscle are equivocal. In atrophied muscles, concurrent decreases in fibre size and myonuclear number maintain the myonuclear domain size [7]. However, decreases in fibre size without decreases in myonuclear number have also been reported in ageing, leading to a reduced myonuclear domain size. Hikida et al. [27] showed that the nucleus-to-cytoplasm ratio was not maintained in untrained elderly men. In an animal model, Brack et al. [28] found the number of nuclei per unit length decreased in ageing animals, which resulted in an increased myonuclear domain size. They also found larger fibres to have more satellite cells than smaller fibres, but during the ageing process this declined. They postulated that a deficit in satellite cells resulted in the inadequate nuclear replacement seen in ageing muscle.

Myonuclear domain size appears to differ between fibre types [29]. Slow fibres have more myonuclei per unit area [30], leading to a small myonuclear domain size [7,23,31]. Slow fibres have higher rates of protein turnover [32] and a higher oxidative capacity (requiring greater levels of protein synthesis) [33]. Fast glycolytic fibres, with lower oxidative activity, have relatively larger myonuclear domain sizes [29,31].

Investigations examining changes in myonuclear domain size in ageing skeletal muscle, at least in slow and fast type muscle in rodents, have revealed an increase in number of myonuclei in slow fibres resulting in a decrease in myonuclear domain size [34]. This is perhaps due to a reduced functional ability of the nuclei in slow fibres of old muscle. It can be hypothesised that ageing muscle could benefit from the addition of new myonuclei with younger attributes.

However, research studies examining the effects of ageing on satellite cells also have been equivocal. During the ageing process, satellite cells may decrease in number [35–37]. Other studies found no reduction in satellite cell numbers in ageing skeletal muscle [27,38]. Similar to aged myonuclei, it appears that satellite cells have a decreased functional ability [39,40]. But more recent research has shown that this is due to a decrement of factors which influence the surrounding milieu, e.g. decreased growth factor levels [41]. This is likely to influence the ability of the satellite cells to proliferate and fuse and thus they are unable to influence many of the changes seen in sarcopenia, particularly changes in the myonuclear domain size.

Unlike acute conditions resulting in muscle atrophy, aging and the development of sarcopenia occurs slowly. Nevertheless, the myonuclei have independent transcriptional ability to respond to local stimuli/signals [42]. In both atrophy and sarcopenia, the molecular control of muscle size involves a major negative regulator of muscle mass, as well as other pathways, albeit to different extents.

1.2. The signalling pathways

The molecular mechanisms underpinning muscle atrophy and ageing remain to be fully elucidated. The next section aims to describe a number of key molecular components and contributors to atrophy and ageing. In this book chapter we will review current accounts of three main contributors – myostatin, ubiquitin ligases MAFbx and MuRF1, and IGF1, AKT-mTOR pathway.

1.2.1. Myostatin/SMAD pathway and atrophy/sarcopenia

Myostatin (growth-differentiation factor 8, GDF8] is a member of the transforming growth factor (TGF)β superfamily and a negative regulator of muscle mass. Myostatin appears to be primarily found in muscle tissue [43]. Animals with a polymorphism in the myostatin gene, that renders it less active, experience hypertrophied muscles. This is seen in "double muscled" cattle [44–46], the significant hypertrophy in a child [47]and myostatin knock-out mice [43], all of which demonstrate significant hypertrophy in skeletal muscles in response to myostatin gene mutation. Even with a normal myostatin gene, if expression is blocked experimentally, there are 13-30% increases in skeletal muscle hypertrophy [48]. In evolutionary terms, it is well known that hominid size was not the crucial factor securing survival. Therefore, adequate control of muscle mass requires that hypertrophy be kept in check, and this negative regulator is myostatin.

Conversely, overexpression of myostatin leads to muscle atrophy [49]. Also, situations of muscle disuse in mammals typically lead to increased myostatin mRNA [50] and protein [51]. Myostatin inhibits muscle protein synthesis and growth as well as satellite cell differentiation [52,53].

Members of the TGFβ superfamily, including myostatin, bind to membrane receptors and trigger the phosphorylation and activation of the SMADs, a family of signal transducers [54]. Myostatin binds to activin type II receptors which results in recruitment of the type I receptor to the type II receptor-myostatin complex [55] and activation of regulatory SMADs (SMAD2 and SMAD3] [56]. These SMAD2 and SMAD3 complex with SMAD4 and together translocate to the nucleus where gene transcription is altered.

Studies investigating changes in myostatin levels with muscle atrophy have reported:

i. Spaceflight: increased myostatin transcript levels after 11 days of spaceflight in mice [57].
ii. Animal models: increased myostatin transcript levels as early as one day after hindlimb suspension [58].

iii. Bed rest in humans: 25 days of bed rest resulted in 12% greater serum myostatin levels [59].
iv. Unilateral lower limb suspension (ULLS) in humans: three days of ULLS led to increased myostatin transcript and protein levels [60].

Ageing muscle, again, has shown differing responses. Some authors have reported no relationship between myostatin mRNA levels and skeletal muscle mass [61,62] while others have shown an increase in myostatin levels along with decreased muscle mass in ageing individuals [63,64].

To unravel the complex differences between atrophy and sarcopenia, it is necessary to understand other molecular pathways influencing muscle mass and how these may interact. Indeed, myostatin is thought to act through a number of pathways including upregulation of ubiquitin-proteasome pathway (including MAFbx and MuRF1]; interactions with FOXO, inhibition of AKT-mTOR pathway (see next sections); as well as inhibition of satellite cells (described earlier).

1.2.2. Ubiquitin ligases MAFbx/MuRF1 and atrophy/sarcopenia

There are various molecular mechanisms involved in muscle wasting including calpain, caspase and ubiquitin mediated protein degradation. It is not within the scope of this chapter to discuss these in detail, however since the ATP-dependent ubiquitin proteasome pathway is the primary degradation pathway of skeletal muscle in response to inactivity and disuse, two components of this pathway must be discussed for their role in the assessment of the molecular process of skeletal muscle atrophy. The ubiquitin proteasome pathway is translationally upregulated during muscle atrophy. It involves E1 ligases which activate ubiquitin E2 ligases that are responsible for transferring the activated ubiquitin to the protein molecule that is then targeted for degradation, but still requires a further control step: the E3 ligases regulate the actual transfer of ubiquitin to the protein. Two important skeletal muscle specific ubiquitin E3 ligases are Muscle-specific RING Finger protein1 (MuRF1] and Muscle Atrophy F-box (MAFbx/atrogin-1].

MAFbx and MuRF1 have been identified as factors functioning to a great extent during muscle atrophy in several models of disuse [65]. MAFbx and MuRF1 were first identified following profiling in mouse atrophy after fasting and immobilisation [65,66]. The expression of both appears to be primarily in skeletal muscle. In knock-out models, animals which cannot make MAFbx and MuRF1 proteins, do not lose muscle mass with denervation [65].

MAFbx and MuRF1 mRNA levels are rapidly activated in numerous models of atrophy and are thought to contribute to the initiation of the atrophy process [67]:

i. Spaceflight: increased MAFbx and MuRF1 gene expression in rodents [57]
ii. Animal models: increased MAFbx and MuRF1 after hindlimb suspension [68]
iii. Unilateral lower limb suspension (ULLS) in humans: three days of ULLS led to increased MAFbx and MuRF1 transcript levels [60]; increased MuRF1 after 10 days ULLS and decreased MAFbx and MuRF1 10-21 days of ULLS [69].

iv. Immobilisation (cast or leg brace) in humans: increased MAFbx and MuRF1 gene expression after immobilization [70,71].

Studies on ageing animals have reported both increased [72] and decreased [73] MAFbx and MuRF1 levels. However, in ageing human muscle most studies report no changes in MAFbx or MuRF1 [eg [74]]. This may be due to the prolonged nature of the ageing stimulus, or the distinct lack of anabolic stimulus.

MAFbx and MuRF1 are regulated by the family of Forkhead box O (FOXO) transcription factors [75]. FOXO1 does not directly increase MAFbx or MuRF1. In rodent muscle, IGF1 which is pro-anabolic, inhibits the upregulation of these E3-ligases [75], however upregulation of FOXO1 blocks this influence of IGF1 and allows for the upregulation of MAFbx and MuRF1.

Nonetheless, human models of atrophy do not show the same relationship of FOXO with MAFbx and MuRF1 [eg [60]]. Therefore, to fully understand atrophy and sarcopenia, and before discussing the mechanisms behind potential interventions, it is necessary to elucidate an anabolic pathway, namely the IGF1-AKT-mTOR pathway.

1.2.3. IGF1-AKT-mTOR and atrophy/sarcopenia

Insulin-like growth factor 1 (IGF1) is a critical growth factor that promotes muscle hypertrophy in skeletal muscle. IGF1 acts through the AKT-mTOR pathway which plays a primarily role in skeletal muscle protein synthesis. IGF1 can stimulate the pathway via PI3K and AKT, but ultimately mTOR is the central governing signalling factor which modulates transcription via p70S6K, 4E-BP1, eIF4E and thus protein synthesis. When the AKT pathway is deactivated this leads to muscle atrophy through FOXO [76]. Animals with knock-out AKT genes have significant atrophy. In physiological models of atrophy the following has been reported:

i. Spaceflight: increased FOXO1 transcript levels, decreased insulin receptor substrate-1 (IRS-1] transcript levels after spaceflight in rodents [57]
ii. Animal models: 10 days of hindlimb suspension also led to decreased phosphorylation of AKT and p70S6K [77].
iii. Unilateral lower limb suspension (ULLS) in humans: no change in AKT-mTOR pathway components after 10 days or 21 days ULLS [78].

As mentioned in the previous section, there is an indirect connection between IGF1 and the E3 ligases. In cell culture, deactivation of IGF1-PI3K-AKT has been linked to increased levels of the ubiquitin proteasome E3 ligases, MAFbx and MuRF1 [79]. It is thought that AKT acts directly on FOXO. When growth stimuli are present, AKT acts to phosphorylate FOXO and the phosphorylated FOXO remains in the cytosol. When growth stimuli are removed, AKT is inactive which causes dephosphorylation of FOXO allowing it to relocate from the cytosol to the nucleus and activate genes involved in cell death, cell cycle inhibition and metabolism. Animals with overexpression of FOXO have reduced muscle mass and this appears to be related to increases in MAFbx and MuRF1.

Alterations in IGF1 signalling pathways in older adults contributes to sarcopenia by means of influencing muscle fibre size, increased loss of myonuclei and increased proteolysis [reviewed in [80]]. Since IGF1 has been shown to control muscle mass in cell culture [81], it's relevance and contribution to age-related changes is fundamental. Indeed, increased total AKT (but not phosphorylated AKT) has been reported in ageing adults depicting a reduction in efficiency of AKT in older individuals [82].

Finally, in keeping with the multi-nucleated nature of muscle fibres and the control of myonuclear domain size, IGF1 also acts directly on satellite cells. It causes satellite cell proliferation and its absence is associated with lower proliferation capacity. Therefore, interventions that focus on the IGF1-AKT-mTOR pathway, will also induce activation of the support-system for addition of myonuclei.

2. Methodological analysis and imaging of skeletal muscle changes with atrophy and sarcopenia (including human and animal models)

2.1. Cell Culture

Cell culture, or *in vitro* methods, allow researchers to manipulate the environment in which the cells will live and grow. The cells are grown under extremely controlled conditions and this allows results to be obtained based on absolute control of the system, homogeneity of the sample and less compounds needed for analysis. Using cell culture models allows removal of confounding variables such as surrounding tissues or the effects of repeated stress on an animal or human. It also reduces the use of animals in research. However, disadvantages of cell culture include the cost of setting up the laboratories, maintaining the cell cultures and adding substances that simulate the controlled physiological conditions to the normal culture media. Cell culture provides a great insight into specific pathways (such as those discussed earlier) with addition of known major controllers such as myostatin, to the media. But this model can also provide evidence of the role of specific proteins and pathways by simple removal of specific molecules or the blocking of these by inhibitors or silencers.

Despite all these advantages, within the cell culture models, the cells will not react exactly as they would inside mammalian organism.

2.2. Animal models

There are a number of well characterized and representative animal models to investigate unloading and disuse atrophy, including hindlimb suspension (discussed above). Investigations using models of muscle disuse have yielded a large and important body of knowledge including details explained in this book chapter. However, it must always be remembered that while animal models provide insight and clarification of some of the complex signalling pathways and interactions which are underway during muscle atrophy and sarcopenia; there are a number of key differences which cast a slight shadow on the direct comparison of animal and human models.

It is consistently observed and acknowledged that influences on protein synthesis and degradation are different in human and animal models, including a faster rate of muscle loss in rodent models and a differing response to unloading in slow and fast fibre types in animals compared to humans [reviewed in [83]]. In most animal studies investigating protein synthesis, the animals had not yet reached maturity and were still in the growth phase (albeit slow growth), which is not directly comparable with adult human beings. The response of animals to changes which occur with ageing is also not identical to that of the human response. A crucial issue that needs to be discussed in this regards, is when the ageing response begins in various animal species because this is key to interpretation of findings in animal studies.

Nevertheless, the benefits of animal studies are the insight which they provide for research development and strategies that will aid in knowledge development leading to clinical studies to prevent or alleviate skeletal muscle atrophy and improve muscle mass in humans, and should therefore not be dismissed.

2.3. Human models

The extensive benefit of studying skeletal muscle loss with human research projects is the direct applicability of the results with the potential to provide extremely useful information for individuals, communities and the greater body of scientific knowledge. Furthermore, with the advent of technologically advanced medical imaging facilities, analysis of skeletal muscle disuse and sarcopenia can be achieved to a greater degree of accuracy than before, and more non-invasively.

Magnetic resonance imaging (MRI): uses radio frequency pulses over a powerful magnetic field to image nuclei of the atoms at any depth inside any body tissue. MRI does not use ionizing radiation. The MRI builds two-dimensional images of internal body structures such as organs, soft tissue and bone, with good contrast between the tissues. MRI can measure loss of muscle mass as well as changes within the muscle such as fat infiltration. A further advantage is that the images can be stored digitally for later comparison of the same subject using the same landmarks.

Dual-energy X-ray Absorptiometry (DXA): provides an accurate measure of bone mineral density, with calculations of fat-free (lean body) and fat mass of an individual. The DXA uses two x-ray beams with differing energy levels which create an image of the density of different body tissues. After skeletal muscle atrophy, individuals will have a reduction in lean body mass and may also have an increased fat mass and alterations in bone mineral density. The advantage of this imaging tool is that these concurrent changes in other tissues can be assessed at the same time. This is highly relevant since skeletal muscle is not the only tissue exposed to the external environmental influences promoting muscle loss.

Computed Tomography (CT): uses x-rays to show cross-sectional (or slice) images of the body. Since the x-ray moves around the body, it provides more detail than a regular x-ray. It can be used for diagnostic purposes such as diagnoses of tumors. With disuse and/or sarcopenia the

CT scan can measure cross-sectional area of the entire muscle or the muscles in a functional group, assess content/quality of the muscle based on protein content/density and measure fatty infiltrations and other changes in skeletal muscle occurring with atrophy and disuse.

Positron emission tomography (PET): looks at how the body functions, rather than the body's anatomy (CT and MRI). After the intake of a radioactive sugar, the PET scan shows the metabolic activity of the body and rapidly growing cells. Cells which consume more of the radioactive sugar will be detected as darker on the PET scan.

Skeletal muscle biopsies, small muscle samples taken from humans using minimally invasive methods under local anaesthetic, can provide substantial insight into molecular pathways and alterations in structural components as well as myonuclei and satellite cells after disuse and the long-lasting effects of sarcopenia (as discussed above).

Together, the benefits and discoveries made and clarified in cell culture, animal models and in human muscle tissue samples, combined with those gained from imaging in more holistic human studies, provide an essential and significant insight into the complex interplay of interactions within skeletal muscle with disuse and sarcopenia. Furthermore, the knowledge gained with these experimental techniques will provide an appreciation and clarification of ways to intervene to alleviate, reduce or treat skeletal muscle atrophy and sarcopenia.

3. Prevention and/or alleviation with nutritional and exercise interventions

Because of the complex interplay of neural, hormonal, physical and nutritional alterations which occur with both atrophy and sarcopenia, as well as the progress being made in understanding molecular and cellular pathways with atrophy and sarcopenia, there are numerous potential interventions which can be, and which have been, investigated to combat, alleviate or benefit skeletal muscle mass with disuse and ageing. A number of these will be discussed here.

3.1. Exercise

3.1.1. Resistance exercise

Resistance exercise increases muscle mass and by increasing the load placed on the muscle activates the AKT-mTOR pathway thereby increasing protein synthesis and reducing muscle atrophy. As little as one bout of resistance exercise has been reported to increase IGF1 gene expression [84]. In young individuals, resistance exercise leads to increased protein synthesis after 2-4 hours [85] and this increase is maintained for 24-48 h in untrained individuals [85].

In models of disuse, such as bed rest and immobilization, resistance exercise alone has been reported to reduce, but not completely alleviate muscle loss. Resistance exercise during 14 days of single leg immobilization was sufficient to preserve quadriceps muscle mass [86] and during bed rest exercise alone reduces loss in muscle mass [87].

Resistance exercise training appears to be the only consistent way to combat sarcopenia. Physical activity can help slow the ageing process and improve the quality of life for elderly individuals. Older adults respond well to load bearing activity and increases in strength, muscle size and function are repeatedly found with resistance exercise interventions even in very old individuals, both community dwelling and nursing home residents. Elderly individuals tolerate relatively high intensity resistance exercise and this shows the greatest responses. However, care should be taken when prescribing resistance exercise for elderly individuals taking into consideration other health complications. Resistance exercise training also restores ageing muscle's ability to increase nuclear domain size within hypertrophy [27]. Resistance exercise is thought to affect satellite cells and have a positive effect on age-related changes in satellite cells and myonuclei [reviewed in [88]].

3.1.2. Endurance exercise

Aerobic exercise such as inflight cycle ergometer in space or endurance exercise on earth helps to maintain and improve cardiovascular function [89], however because it has no mechanical loading, endurance exercise has no large effect on skeletal muscle mass and therefore does not reduce muscle atrophy or sarcopenia. Furthermore, the intensity of the endurance exercise must be sufficient to maintain aerobic fitness [90]. Since endurance exercise does show positive influences by enhancing muscle oxidative capacity in elderly individuals, combining an endurance modality with resistance exercise may add further benefits to the muscle.

3.2. Pharmacological

3.2.1. Testosterone

Testosterone has been shown to regulate skeletal muscle mass in humans [91] and anabolic steroids are known to increase muscle mass in healthy individuals by increasing protein synthesis and accelerating activation of satellite cells and interestingly administration of androgens increases satellite cell numbers in a dose-dependent manner [92,93]. This response is particularly noted when combined with load bearing exercise. However, physiologically relevant increases in testosterone do not appear to enhance protein synthesis following resistance exercise [94].

During sarcopenia, decreased circulating levels of testosterone and dehydroepiandroste (DHEA) are prevalent [95]. Studies have reported a relationship between serum testosterone levels and muscle strength in older men. Testosterone alone has shown equivocal results in elderly individuals. Increases in lean body mass, but not knee extensor strength has been reported [96] However, increases in muscle strength, muscle fibre size and satellite cell response after 20 weeks testosterone supplementation are promising in humans [97] and mice [98].

3.3. Nutritional

3.3.1. Protein/essential Amino Acids

Protein synthesis and degradation are influenced by nutrient intake and intake of proteins and amino acids (AA) stimulate muscle protein synthesis and inhibits protein breakdown [99]. In particular, leucine, an essential amino acid, is an especially powerful stimulator of protein synthesis by both insulin dependent and independent pathways. Carbohydrate and protein are known to stimulate protein synthesis and can positively influence IGF1-mTOR-AKT pathway to stimulate protein synthesis and prevent upregulation of FOXO, MuRF1 and MAFbx; therefore nutritional intake could be a powerful countermeasure in reducing muscle mass loss with disuse, particularly in situations where exercise is not feasible (such as hospitalized bed rest).

Essential AA supplementation has been consistently shown to influence protein synthesis and alleviate some, but not all, of the loss of skeletal muscle lost with bed rest [100], but not to the same extent as exercise. However, 28 days of immobilization with protein and amino acid supplementation [28g protein) did not prevent increases in myostatin, MuRF1 or MAFbx over time [101].

The post-absorptive rate of protein synthesis is similar in young and elderly individuals but elderly individuals may have a delayed anabolic sensitivity to amino acids compared to young individuals [102]. However, elderly individuals can still stimulate muscle protein synthesis with increased protein intake, and it also must be noted that many elderly individuals do not take in the recommended protein intake and this may contribute further to muscle mass loss [103]. Other nutritional interventions have produced positive results against sarcopenia, in particular, high quality protein diets may be beneficial for stimulating protein synthesis in the muscles of ageing individuals [104].

3.3.2. Creatine

Creatine supplementation has been reported to increase energy production, increase fat free and muscle mass. It has been reported to improve recovery after immobilization and to act, at least in part, through myogenic regulatory factors which influence satellite cells [105] and is therefore of interest to reduce muscle loss with clinically related atrophy as well as of potential interest in combatting sarcopenia with the elderly population.

However, older individuals appear to respond differently than young individuals to creatine supplementation [106]. Short term creatine supplementation in older adults has shown mixed results ranging from no effect [107–109] as well as increased anaerobic power and work capacity of sedentary older adults [108]. Short term creatine supplementation also has produced increases in strength and fat free mass of elderly men and women [110,111]. In longer studies, neither creatine supplementation, protein supplementation, or a combination of protein and creatine was shown to provide further benefit beyond 16 weeks of isotonic resistance training alone in elderly subjects [112]. However, a recent review on creatine

supplementation and elderly individuals suggests that timing and dose may also play a critical role in response to supplementation [113]. Since creatine supplementation is a natural, inexpensive and as far as we know, safe supplementation; and since creatine supplementation may provide other benefits to elderly individuals such as improved cognitive ability [114] it continues to be a potential factor in interventions to alleviate sarcopenia.

4. Summary

In summary, this book chapter has aimed to summarise skeletal muscle changes with disuse atrophy and sarcopenia, which are both significant public health issues and potential burdens on society. This chapter has introduced and illustrated cellular and molecular changes which occur within skeletal muscle, particularly focusing on myonuclei, satellite cells, and various relevant signalling pathways including - myostatin/SMADs, ubiquitin proteasome pathway and IGF1-AKT-mTOR pathway. The full interactions and molecular underpinnings remain to be elucidated, but we have reviewed the current knowledge and accounts of these three main contributors. These pathways are complex, and furthermore interact with each other in important ways to modulate muscle protein synthesis, muscle degradation and maintenance/loss of muscle mass. The significant impact of varying and advanced methodological analyses have been reported and the influence of molecular analysis, cell culture models, animal models as well as tools to measure interventions in human research have been reviewed. Finally, a number of preventative strategies have been discussed which are promising for minimising muscle atrophy and sarcopenia. The combination of nutritional and pharmacological interventions combined with exercise are favourable, practical and feasible applications to successfully influence prevention and alleviation of atrophy and sarcopenia. Understanding the disuse-related changes and age-related declines in muscle mass will provide insight into preventing and combatting sarcopenia. This will benefit by improving quality of life in the increasing numbers of elderly individuals as well as reducing the burden on the community and the healthcare system.

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Chapter 9

Signaling Pathways that Mediate Skeletal Muscle Hypertrophy: Effects of Exercise Training

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Additional information is available at the end of the chapter

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1. Introduction

Skeletal muscle accounts for approximately 50% of total body weight, and is known to be the largest tissue in the human body, mainly responsible for force generation, movement and breathing [1,2]. The muscles consist of four main types of fibers, type 1, 2A, 2D/X and 2B, which differ in their contractile and metabolic properties. This difference is dependent on the myosin heavy chain (MHC) isoform that predominates in each fiber type and the gene expression program, and therefore the distribution of fiber types is genetically determined [2,3].

The various muscle functions are controlled by signaling pathways that allow the muscle fiber respond to changes in the metabolic and functional demands of the body. Indeed, examples in the world of sports, therapy, surgery, and trauma support the idea that skeletal muscle is one of the most adaptable tissues in the body.

Skeletal muscle response varies whether the level of use increases or decreases. In fact, muscles are always trying to tailor their molecular, structural and functional properties to the level of use demanded of them. However, there are situations which the level of use, metabolic load, or the level of stress on a muscle fiber is so great that the fiber suffers damage, in which part of the muscle cell degenerates and is replaced with new muscle tissue. Such a response has important implications for the normal development process, the potential use of regenerating muscle in treatment of muscle disease and in sports performance [4].

Recently, a large number of studies have suggested that some diseases such as cancer, diabetes and AIDS [5,6], and unfavorable environmental conditions, such as immobilization and fasting can lead to reduction in skeletal muscle mass, known as

muscle atrophy [7]. In contrast, some forms exercise training, such as strength training and resistance training, can produce an increase in skeletal muscle mass, known as muscle hypertrophy [8]. These interactions suggest that the dynamic regulation of skeletal muscle mass is not simply a balance between synthesis and protein degradation, but a finely regulated process.

Skeletal muscle hypertrophy is known to increase the cross-sectional area of skeletal muscle by biosynthesis of new structures involved in muscle contraction, known as one of the main alterations generated in the muscle as a result of exercise training [9,10].

The development of molecular biology techniques have allowed major advances in understanding the intracellular signaling pathways responsible for regulating skeletal muscle tropism and their adaptations to different types of exercise training. Thus, several studies have recently been published addressing this topic with great richness of detail [1,9-12].

The synthesis of new contractile units takes place by known processes, and studies of information about gene flow have shown that this begins with the replication, maintenance and rearrangement of DNA, through synthesis and RNA processing (transcription) and culminating with the synthesis and processing of regulatory proteins (translation) [13,14]. These processes are sequential, capable of regulating skeletal muscle mass at various points and in response to a chronic stimulus, such as exercise training. They can lead to an over-compensatory response to this stimulus resulting in the formation of new contractile muscle units, which will lead to increased muscle size and strength. This remodeling that occurs in skeletal muscle involves intracellular signaling pathways and consequent gene reprogramming that results in changes in mass, contractile and metabolic properties.

The main pathways responsible for a biochemical cascade of intracellular signaling will be addressed in this review with purpose of providing an integrated view of processes that promote the increase or decrease the size of the muscle fibers resulting from exercise training. Therefore, various intracellular signaling pathways involved in regulation of skeletal muscle mass induced by exercise training have been reported in the literature, and the main focus of this chapter was to review processes, such as the Akt-mTOR pathway, myostatin and microRNAs (miRNAs).

2. Signaling pathways involved in muscle remodeling

Skeletal muscle responds to physiological stimuli such as exercise training, and remodels to adapt to new demands imposed by this stimulus. This adjustment is made by extracellular stimuli to reach the cell membrane and interact with receptors activating intracellular signaling pathways, which result in changes in gene transcription and protein synthesis and thus promote muscle remodeling. In this review, some of the most important intracellular pathways are presented.

3. Exercise training and the Akt-mTOR pathway

Protein synthesis is regulated at several levels and involves a complex biological network of intracellular signaling mechanisms. The signaling pathway IGF-1/PI3K/Akt (growth factor like Insulin-1, phosphatidylinositol 3-kinase and protein kinase B, respectively) is considered the main mediator of normal muscle development and one of the most studied signaling molecular systems involved in muscle hypertrophy. This pathway plays a key role in the hypertrophic process, since it coordinates the molecular basis related to protein degradation and synthesis [7,9,10,15-21].

The Akt family is composed of three members: Akt1 (PKB-α) Akt2 (PKB-β) and Akt3 (PKBγ). These three isoforms share over 80 % homology and are expressed in a tissue specific manner, thus the Akt1 and Akt2 isoforms are predominantly expressed in skeletal muscle, the brain, heart and lungs and Akt3 is more expressed in the brain and testicles [22,23].

There are various stimuli that lead to the activation/phosphorylation of Akt: such as growth factors [24], cytokines, hormones, which occurs in a manner dependent on phosphatidylinositol 3 kinase (PI3K) [25], suggesting that Akt plays an important role in mitogenic cellular function and protein synthesis. In fact, studies with knockout transgenic mice for Akt1, have shown deficiency in muscle growth [26] and mice that overexpressed Akt1 have resulted in a hypertrophic skeletal muscle phenotype [27]. Specifically in skeletal muscle, expression of the active isoform of Akt1 results in *in vitro* and *in vivo* myotube hypertrophy, and also prevents atrophy in denervated muscles [7].

Another stimulus capable of inducing components of this pathway independently of agonists is called mechanotransduction, which consists of conversion of the mechanical signal into a biochemical event, and it also plays a key role in inducing protein synthesis. Thus, mechanical signal transduction is also capable of inducing growth by means of a mechanism other than growth factor signaling, which is independent of upstream elements such as IGFI and PI3K [28]

The strength training consists of mechanical stimuli and is a potent agent that increases tropism in the skeletal muscle. This increase is triggered by increase in IGFI or MGF (mechano growth factor) protein expression which leads to a sequential activation cascade, ordered by PI3K, PDKI and II (phosphoinositide dependent kinase I and II) and Akt. After this, Akt promotes activation of two independent pathways: mTOR (mammalian target of rapamycin) and GSK3β (glycogen synthase kinase-3β) that play a crucial role in skeletal muscle hypertrophy [18].

PI3K is an enzyme highly expressed in skeletal muscle and its primary activity is the phosphorylation of some lipids (phosphatidylinositol) in position 3 of the inositide group (D3) [29]. Activation of Akt induced by strength training is a process that involves several steps and additional proteins. The activation of PI3K by IGFI/MGF results in phosphatidylinositol 3 phosphate (PIP3), which leads to translocation of Akt to the membrane and a conformational change that allows PDKI and PDKII to phosphorylate the

Ser473 and Thr308 residues activating Akt [30]. There is evidence that Thr308 is phosphorylated by PDKI and that the Ser473 residue can be phosphorylated by Akt, PDKII or other kinases that have not yet been discovered [30]. Once activated, Akt phosphorylates mTOR and GSK3β, which mediate protein synthesis, transcription and proliferative processes related to hypertrophy response, as well as control of protein degradation [10].

The mTOR functions as a central integrator of a wide range of signals that modulate protein metabolism and cell growth. There is evidence that phosphorylation of mTOR induced by strength exercise training acts on protein translation on a global scale, since three downstream components of this pathway: $p70^{s6k}$, 4E-BPI and eEF2 facilitates initiation of the translation process, mainly of mRNAs with complex secondary structures in the 5´untransated region, promoting biogenesis of ribosome, respectively. $P70^{S6K}$ stimulates protein synthesis due to its action both on mRNA translation which has oligo-pyrimidine sequences in its 5´UTR region adjacent to CAP (m7GpppG) and also on the phosphorylation of the ribosomal peptide S6 by kinase $p70^{S6k}$ [15, 31].

Moreover, mTOR directly phosphorylates the protein 4E-BPI/PHAS-I. Once phosphorylated, it releases its inhibitory effect on the translation initiation factor elF4E, which impairs inhibition of translation initiation by coupling with the end CAP of mRNA. There is one last active effect of mTOR on increasing muscle mass that consists of its effect of decreasing phosphorylation of S6K kinase, leading to the increase in cross-sectional area of skeletal muscle [15,31].

The first evidence that mTOR and its activation of $p70^{S6K}$ could play a role in the mediation of hypertrophic effects induced by strength training was shown by Baar & Esser [32]. In this study the phosphorylation of $p70^{S6k}$ was increased in the tibialis anterior and extensor digitorum longus in 3 and 6 hours after the strength training session. Thus, the direct role of mechanical overload on the activation of this ribosomal unit was shown, as well as a direct correlation between the increase in $p70^{S6k}$ and the increase in muscle mass induced by strength training.

Furthermore, definitive studies were conducted on the signaling mechanisms of mTOR in skeletal muscle hypertrophy, induced by strength training, with the use of rapamycin, a specific mTOR inhibitor. Some studies [33,34] used Sprague Dawley rats submitted to one strength exercise session, to assess the acute effect of this type of exercise. Increased protein synthesis was found in the gastrocnemius muscle 16 hours after the session, which was completely prevented by administration of rapamicyn used 2 hours before of the exercise session. In contrast to these results, recent studies have shown that aerobic exercise training increased the phosphorylation of protein kinases activated by AMP (AMPK), which directly phosphorylated TSC2 also known as Tuberin or Tuberous Sclerosis protein 2. This led to the inhibition of mTOR, which suggests that protein synthesis is inhibited in this type of training [18].

Furthermore, Akt is related to another pathway parallel to mTOR, which induces hypertrophy through phosphorylation in Serine 9 of GSK-3β [35]. When phosphorylated, GSK3β is inhibited, decreasing eIF2B in Serine 535 activity, which promotes the translation

initiation process [36], In fact, studies have reported the increase in GSK-3β phosphorylation, which leads to eIF2B inhibition immediately after and 3 hours after a strength training session, supporting hypothesis that this pathway is also involved in the stimulation of protein synthesis induced by strength training [18,37].

Although the role of this pathway in strength exercise-induced hypertrophy has been established, the contributions of individual pathways regulating mTOR during mechanical overload-induced skeletal muscle hypertrophy are poorly defined and there are several controversial results related to exercise. A recent study showed that 10 days of mechanical overload induced progressive hypertrophy of the plantaris muscle and this growth was associated with significant increases in total RNA content and protein metabolism in C57BL/6J mice. Inhibition of PI3K activity by wortmannin was sufficient to block insulin-dependent signaling, but did not prevent the early activation of mTOR in response to overload. Akt phosphorylation and GSK3β, were not significantly increased until 2-3 days of overload had occurred. In contrast, mTOR was activated after a single day of overload as indicated by a significant increase in S6K1 phosphorylation [38]. In addition, the mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK)-dependent pathway was activated at day 1 after overload, which provided evidence that the MEK/ERK pathway may contribute to mTOR activation through the phosphorylation of TSC2 [38]. However, it is not clear whether this is the main mechanism that activates mTOR [39]. This study demonstrated an independent activation of $P70^{S6k}$ and cross-talk between MEK/ERK and mTOR pathways that may provide elucidation about skeletal muscle growth in response of exercise training in future.

A third important function of Akt in skeletal muscle tropism is the regulation of the transcription through inactivation of Forkhead Transcription Factors, also called FOXO or FKHR, which are responsible for gene transactivation involved in components of the proteolytic system coordinated by the ubiquitin-proteasome system [40,41].

Three FOXO isoforms have been investigated and have been well characterized FOXO-I, FOXO-3a and FOXO-4 [42]. The FOXO isoforms are predominantly located in the nucleus where they are activated. However, when they are phosphorylated, mainly by Akt protein, these FOXO proteins are extruded to cytosol, and they are not capable of inducing the transcription of genes involved in muscle atrophy, such as atrogin-I/MAFbx and MuRF, two specific muscle E3 ligases, that are components of the ubiquitin proteasome system [43-45]. Studies have shown that when Akt pathway signaling is inhibited there is an increase in atrogin-I transcription, and also inversely, FOXO-I expression is reduced in hypertrophy [16,17]. These results strongly suggest that that skeletal muscle hypertrophy induced by strength training in healthy individuals is at least partly related to FOXO-I inhibition by Akt.

The effect of detraining was also studied, and inversely to that which was observed in response to strength training, the signaling described for Akt phosphorylation decreased [17] and there was a decrease in GSK3β phosphorylation, which emphasizes that this pathway is also involved in the skeletal muscle atrophy process induced by the interruption of strength training.

In addition, there is evidence that the activation of this pathway is dependent on age, gender, variations in the type and intensity of the exercise performed, mode of contraction (concentric/eccentric). Moreover, differences in type I and type II fibers activated in force development influence the phosphorylation of these key signaling proteins [32,46,47].

Studies have shown that strength training is capable of activating the Akt pathway acutely and chronically, in a predominant and specific manner when compared with endurance training [17-19;48]. However, there are studies showing that it is a key pathway to cardiac physiological hypertrophy induced by swimming exercise in mice, which also suggests a tissue dependent activation [49] (Figure1).

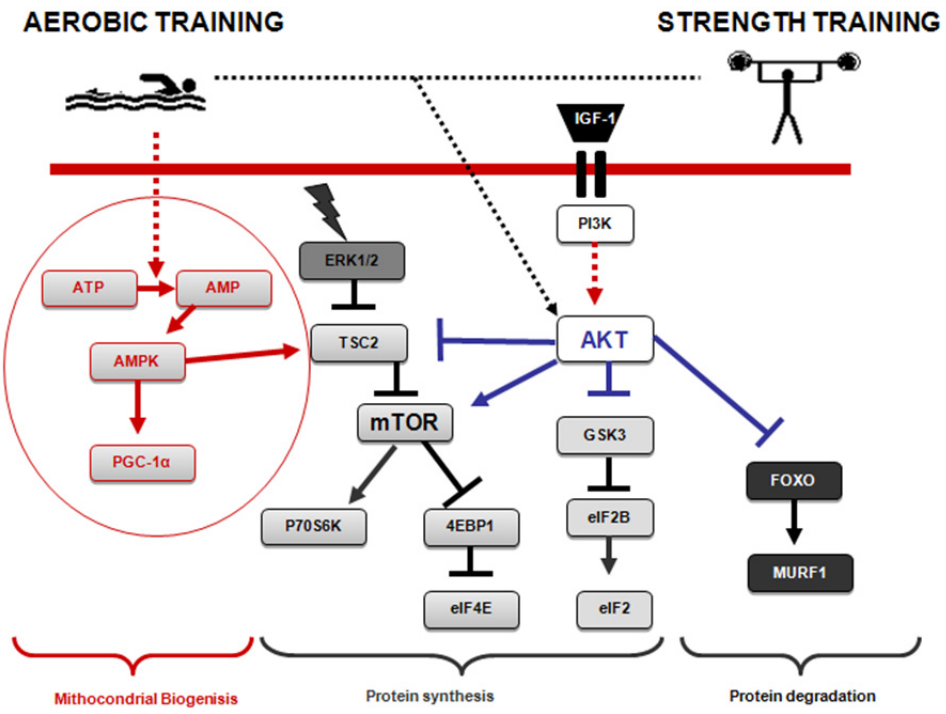

Figure 1. Signalling pathways regulated by exercise and/or IGF-I. Exercise has been shown to activate Akt and ERK1/2 directly, and also induces IGF-I synthesis in muscle. PI3K/Akt is of particular importance as it increases protein synthesis via mTOR activation and inhibits protein degradation via the inactivation of FOXO transcription factors with resistance training. AMPK-activation leads to PGC-1α activation and induces mitochondrial biogenesis with endurance training. ERK = extracellular signal-regulated protein kinase; mTOR = mammalian target of rapamycin; TSC2 = tuberous sclerosis complex 2; PI3K = phosphatidylinositol 3-kinase; eIF2 = eukaryotic initiation factors 2; eIF = eukaryotic initiation factors, including 4E and 2B; 4EBP1 = 4E binding protein 1; ATP (adenosine triphosphate) AMPK = AMP (adenosine monophosphate)-activated protein kinase; MuRF1=muscle RING finger 1; FOXO = Forkhead box; PGC-1α= Peroxisome proliferator-activated receptor-γ coactivator 1α.

Strength/resistance training and some forms of intermittent high-intensity stimulation have been associated with an increase in protein synthesis and thus substantial muscle hypertrophy and gains in maximal force output, whereas endurance training does not promote skeletal muscle hypertrophy or increase the force-output ability of muscle [50]. Human subjects were divided into groups performing endurance training and strength training in a single-bout of exercise, and non-exercised control group. The protocol was conducted with an exercise mode in which the exercise subjects were accustomed during a prior training period of 10 weeks. Activation of several components of mTOR and its downstream signaling were activated exclusively by strength training, while AMPK and its substrate were activated only in endurance trained individuals. Whereas, the strength training produced a non significant increase in phosphorylated AMPK and did not increase activation of its substrates. It is known that AMPK activation may phosphorylate raptor, a mTOR complex component, and it has been suggested that this may switch off mTOR activity [51,52]. However, there have also been studies that have shown an increase in the Akt-mTOR pathway with endurance training [53]. It has been suggested that AMPK activation induced by endurance training also reduces energy consumption related to the protein synthesis process in situations that combine energy consuming exercise and fasting [51].

In rats, AMPK *vs.* Akt-mTOR signaling divergence has been suggested to explain the conversion of endurance training stimulation into mitochondrial biogenesis and conversion of resistance training stimulation into muscle hypertrophy, respectively [18].

Research using experimental animals has indicated that type I and type II fibers might respond differently to contractile activity [19]. The increase in muscle size produced by regular performance of resistance exercise is largely due to an enhancement in the size of type II fibers [54]. Recreationally active male subjects performed four sets of six maximal lengthening contractions with one leg. Muscle biopsies were taken from the vastus lateralis before and immediately after exercise. After 1 and 2 hours of recovery, the elevation of $p70^{S6k}$ and the reduction in eIF2 phosphorylation in the type II fibers after resistance exercise suggest the stimulation of protein synthesis, which may contribute to a more pronounced enlargement of these fibers. In reference [21] it was observed that both concentric and eccentric contractions resulted in extensive phosphorylation of mTOR and $p70^{S6k}$ in the fast-twitch muscles of the rat, while no effect on the slow-twitch soleus muscle was detected after concentric contractions.

The mode of contraction, particularly in eccentric (lengthening) exercise has been considered important for muscle growth, due to inducing a greater amount of muscle fiber enlargement than concentric exercise after a period of immobilization [55]. In addition, at maximal intensities, protocols involving lengthening exercise stimulated myofibrillar protein synthesis more rapidly than shortening exercise [56]. In contrast, other studies have shown similar increases in muscle growth after concentric and eccentric exercise training, accompanied by the same increase in the rate of protein synthesis after exercise [57,58].

There is evidence that eccentric contraction induces a greater magnitude of hypertrophy than concentric contraction. A time course study conducted in the absence of nutritional supply [59] compared the effect of training stimuli of eccentric and concentric contraction modes on Akt/mTOR/$p70^{s6k}$ activation in human males, and reported that the eccentric contraction protocol resulted in greater activation of this pathway than the concentric protocol. Ten subjects performed four sets of six maximal one leg concentric, versus four sets of six maximal eccentric exercise of equal force on the other leg. Although there were no significant changes in phosphorylation of Akt and mTOR, the maximal eccentric protocol induced two to eightfold increases in phosphorylation of $p70^{S6k}$ and in ribosomal protein S6k, which persisted for two hours into recovery period. On the other hand, the concentric protocol or submaximal eccentric contractions did not increase phosphorylation in Akt and mTOR, and there was no phosphorylation in $p70^{S6K}$ and S6k up to two hours later. The authors suggested that an enhanced hypertrophic response to this mode of contraction may occur due to maximal eccentric contraction being able to activate $p70^{S6k}$, independently of the Akt pathway, in addition to being more effective in simulating protein synthesis, an effect that can be induced by the combination of greater tension and stretching of the activated muscles. Indeed, the peak force produced by the eccentric contraction mode is greater and thus results in a increased force per active fiber, increasing mechanical stimulus to the muscle fiber which is known to activate this pathway [60,61].

Recently, the influence of eccentric contraction velocities on Akt pathway and MGF expression was studied. The effect of eccentric contraction velocity manipulation on vastus lateralis hypertrophy was investigated in a study with twenty human males. The subjects performed five sets of 8 repetitions of a slow or fast eccentric protocol and biopsies were collected at the following time intervals: baseline, immediately after, and two hours after the session. MGF expression was increased approximately 2.5 fold in slow eccentric contraction, and Akt and $p70^{s6K}$ protein phosphorylation were higher in the fast eccentric protocol than at baseline, or slow or fast eccentric contraction, which suggests that the velocity has no direct influence on the activation of this pathway, and other signaling pathways could be selectively activated and involved in the response to high velocity eccentric contraction [62].

As skeletal muscle adaptation to exercise is highly dependent on the specificity of training performed, is interesting pay attention to concurrent training effects on this signaling pathway. The effects of a combination of one bout of endurance followed by strength exercise (concurrent training) on this Akt-mTOR pathway were studied by Wang et al. [53], who assessed whether resistance/strength exercise could change the molecular signaling response to endurance training in skeletal muscle. Although the authors had hypothesized that strength exercise would induce a downregulation of genes related to oxidative metabolism, strength exercise performed after the endurance exercise induced an increase in the expression of PGC-1α, PGC-1-related coactivator (mitochondrial biogenesis genes) and PDK4 (aerobic substrate regulation marker), one and three hours after completion of the exercise bout. AMPK phosphorylation was increased to a similar extent between the endurance and resistance/strength exercises at one hour post-exercise but was reversed at three hour post-exercise without difference between endurance and strength training [53].

The acute molecular response to concurrent exercise was investigated in a protocol in which the individual performed resistance exercise either before or after endurance exercise and there was no increase in the phosphorylated form of AMPK, and only a modest but not significant increase in PGC-1α mRNA [63]. It was not clear if this different finding was due to interference from resistance exercise, or the change in the order of exercise, or whether the stimulus was low considering the training status of the subjects. Thus the controversy about whether adaptation to endurance training can be affected by the addition of resistance force remains, since both protocols used strength exercise. Although the consecution of resistance exercise seems be an enhancer of the acute effects of endurance training in the first study, whereas the order of stimuli was inverted only in the second study, the absence of difference could be due a similar number of stimuli.

Protein synthesis and muscle adaptation are regulated in a different manner with aging in different muscle types and genders. Age related atrophy or sarcopenia is thought to be a consequence of normal aging and it is characterized by decreased muscle strength, reduced performance and a decreased capacity to induce hypertrophy after an increase in muscle loading [64]. The Akt-mTOR signaling pathway and its important components associated with the induction of muscle hypertrophy are attenuated with aging [21]. Decreased mTOR, $p70^{S6K}$, 4E-BP1, and ribosomal protein S6 phosphorylation have been reported after 7 days of muscle overload in aged animals in comparison with young adult animals [65,66].

Little is known about gender-based differences in muscle protein synthesis [67]. It has been shown that muscle protein synthesis is similar in healthy young men and women and that resistance exercise induced increase in muscle protein synthesis and mTOR signaling irrespective of sex. A recent study [68] that examined the acute response to resistance exercise in leg muscle, showed that protein synthesis was significantly increased by 52% in young men and by 47% in young women during the first 2 hours of post-exercise recovery.

In conclusion, there are many results that explain the mechanisms involved in regulating the increase in muscle mass induced by exercise training. The Akt-mTOR signaling pathway is capable of coordinating anabolic and catabolic pathways, leading to an increased hypertrophy or atrophy response, which can contribute to elucidating the importance of exercise training in future pharmacological and clinical interventions.

4. Exercise training and myostatin

Myostatin, also called growth and differentiation factor-8, is a member of Transforming Growth Factor-beta superfamily (TGF-β) that functions as a regulator of muscle mass [69,70]. Myostatin expression is identified during the early stages of embryogenesis and continues to be expressed during development of skeletal muscle. In later stages and in adult animals, myostatin is predominantly expressed in skeletal muscle and adipose tissue. However, using a more sensitive real time-PCR techniques, myostatin transcripts could also be detected in the heart and mammary tissues [71].

Several studies showed that myostatin overexpression reduce muscle mass, fiber size, and myonuclei number [72,73]. On the other hand, blockade of myostatin resulted in excessive growth and increased force generation of skeletal muscle indicating that this member of the TGF-β superfamily is a negative regulator of skeletal muscle hypertrophy [69,70,74,75]. Studies with myostatin knockout mice showed increased body weight of approximately 30% in the young adult ages (2–5 months) related an increase in muscle mass. Compared to the wild type, the pectoralis muscles of the myostatin knockout mice increase up to 262%. This increase in muscle mass was due to the hyperplasia or increased number of muscle fibers (82% increase in the tibialis anterior muscle fibers) as well as hypertrophy or increase in the cross-sectional area of individual fibers (14% in tibialis anterior fibers muscle and 49% in the gastrocnemius fibers muscle) [69,70,75]. Also, when myostatin knockout mice were examined at an older age (9 month), they still revealed excessive muscle size however the total body weight had been normalized compared to the wild type animal. The body weight normalization despite the presence of excessive muscle mass was due to the deficit in body fat development. Decreased fat accumulation in myostatin knockout mice resulted from a decrease in number as well as size of adipocytes [74].

To study the mechanisms of action of myostatin on skeletal muscle development, most investigators treated muscle precursor cells with recombinant myostatin protein. A number of studies have been performed in vitro on C2C12 myoblast cell lines and in vivo during chick embryonic muscle development, which showed that myostatin prevented proliferation and differentiation of muscle cells precursors [76,77]. Myostatin induced expression of the cell cycle inhibitors p21 [78] and inhibited expression of myogenic regulatory factors, which encode transcription factors regulating muscle differentiation [79]. Conversely, lack of myostatin should stimulate proliferation and differentiation of muscle precursors. In agreement with this hypothesis, McCroskery et al. [80] showed that satellite cells, normally a quiescent muscle stem cell population required to repair and regeneration of adult muscle, were increased in number relative to the myonuclei of the muscle fibers and showed increased proliferation rates in myostatin knockout mice. In contrast, there are studies which these satellite cells did not proliferate more than those from wild type animals [75].

The myostatin gene encodes a small signal sequence at the N-terminus followed by a large pro-peptide region (also called the latency association protein or LAP-fragment) and a smaller mature region at the C-terminus [69]. The signal sequence is required for processing and secretion. The pro-peptide region regulates the biological activity of myostatin. The mature region binds to one of the two Activin type II receptors (ActRIIB to a greater degree than ActRIIA) a family of serine/threonine kinase transmembrane receptors on target cells [70]. Binding of the ligand to ActRIIA/B, leads to the phosphorylation and activation of the Activin type I receptor, which in turn initiates the intracellular signalling cascade by phosphorylating the receptor-regulated proteins Smad2 and Smad3. Upon phosphorylation Smads form heterodimers with a Co-Smad, Smad4, and these activated Smad complexes translocate from the cytoplasm to the nucleus where they regulate transcription of target genes [79,81] (Figure 2).

Myostatin is found as inactive complex in plasma and muscle tissue of humans and mice, and the action of proteases making it active by cleavage of the pro-peptide region releasing the mature region [82]. Interestingly, Yang et al. [83] created transgenic mice that overexpressed the myostatin propeptide under the control of the Myosin Light Chain (MLC) promotor. In this study resulted in an increase of carcass weight of 48% at 18 weeks. Morphometric analysis revealed an increase in fiber diameter but no in fiber number. The ability of myostatin to inhibit muscle growth may therefore not necessarily rely solely on gene transcription but could also be reliant upon its availability from the extracellular matrix. This proposes a link between the integrity of the extracellular matrix to myostatin activity. This point is significant since there is extensive remodeling of the extracellular matrix during muscle degeneration/ regeneration increasing growth regulatory proteins synthesis [75].

Although the mechanisms of activation are not well known, specific factors also are responsible for generating the active species and subsequent inhibitory activity of myostatin [84]. The following myostatin binding proteins have been identified to date: Activin Receptor, Follistatin and the Follistatin related proteins FLRG (Follistatin Related Gene) and GASP-1 (Growth and Differentiation Factor-Associated Serum Protein-1). In addition, specific antibodies have been developed which bind and inactivate myostatin [70,75,84,85].

Lee and McPherron [70] have created transgenic mice expressing a dominant negative form of the Activin II receptor. This was achieved by expressing a truncated form of the receptor that lacked the intracellular kinase domain.Therefore, although the truncated receptor would still be able to bind myostatin, it would not be able to activate the signal transduction cascade. Over-expression of the truncated form of the receptor led to significant increases in muscle mass, which resulted from hyperplasia (27%) as well as hypertrophy (19%). Whilst not suitable in a clinical setting, the genetic approach does highlight that the Activin receptor could be targeted to promote muscle development through the development of receptor inhibitors. However, it is important to emphasize that the presence of non-functional Activin receptor leads to infertility [85].

The follistatin is expressed in different tissues and acts as an antagonist of different family members TGF-β [86]. A study in knockout mice for the gene of follistatin observed excessive loss of muscle mass [85]. On the other hand, follistatin overexpression mice showed 327% increase in muscle mass compared to control group [70]. The excessive increase in muscle mass observed in these mice resulted from the combination of muscle hypertrophy (27%) and hyperplasia (66%). Recent studies show a high affinity and direct interaction of follistatin to myostatin [87], suggesting its direct action in controlling the activity of myostatin. Remarkably, the increase in muscle mass was far greater than that observed following the deletion of the myostatin gene [69]. One possible explanation for these differences is that follistatin is likely to antagonize molecules in addition to myostatin that also act as muscle growth inhibitors. Introducing a structure homologous to follistatin, the FLRG can also play an important role in the regulation of myostatin [88], by binding to its mature region and inhibiting its biological activity.

Other possible myostatin inhibitor is the GASP-1, which contains domains that are serine protease inhibitors. The GASP-1 interacts with both regions of the myostatin negatively regulates its activity by inhibiting the activity of proteases on the myostatin preventing the release of the mature region [89].

Several studies showed mutations in the myostatin gene related to skeletal muscle hypertrophic phenotype. Double muscling is a trait previously described in several mammalian species including mice [90], cattle [91,92], sheep [93], and human, the latter described once in a German boy [94] and is caused by mutations in the myostatin gene. Mosher et al. [95] have discovered a 2-bp deletion in the whippet myostatin gene that in the homozygote state results in a double-muscling phenotype commonly referred to as the ''bully'' whippet. This deletion causes a premature truncation of the protein, removing the latter 17% of the protein. The whippet breed was developed in the late 1800s specifically for the sport of racing. Despite its comparatively small stature it is a very fast dog capable of running up to 35 miles per hour [95]. Studies of muscle composition in myostatin knockout mice demonstrate a higher proportion of both fast type II and glycolytic fibers, versus slow type I and oxidative fibers when compared to wild-type mice [96]. In addition, the glycolytic muscle phenotype of myostatin knockout mice is associated with a decrease in capillary density, mitochondrial number and expression of mitochondrial enzymes [69,97]. While this change in muscle composition may offer an advantage to whippets, which typically race a short sprint of 200-300m, it may be disadvantageous to whose races extend to 900m and where endurance is more important.

These findings have implications for competitive and professional sports. Studies show that a disruption in the function of the myostatin gene can increase an individual's overall athletic performance in a robust and measurable way. To date, the muscular hypertrophy phenotype has been described in a single human child [94]. This child possessed two copies of a G-to-A transition in the noncoding region of the human myostatin gene. This mutation results in the mis-splicing of precursor mRNA, which most likely truncates the myostatin protein. The child's mother, a former professional athlete, was heterozygous for this mutation and also appeared muscular, although not to the same degree as her child. As discussed by several authors, human athletes could undergo so-called gene doping via disruption of myostatin. The potential to increase an athlete's performance by disrupting myostatin either by natural or perhaps artificial means could change the face of competitive human and canine athletics. Given the poorly understood consequences for overall health and well-being, caution should be exercised when acting upon these results [95].

Interestingly, recent studies show that the increase in muscle mass induced by exercise training may be related to the regulation of myostatin [98]. Treadmill-trained rats showed decreased expression of myostatin in gastrocnemius and vastus lateralis muscles, showing that exercise training is effective in reducing the levels of this protein [99]. However, a study comparing the effects of endurance exercise and resistance training on the expression of myostatin showed different times of myostatin gene expression between the modalities. The aerobic training reduced the expression of myostatin from 8-12 hours after the session, and

this effect was less pronounced when compared to resistance training, where the reduced expression of myostatin was observed 1-24 hours after the training session [100] (Figure 2).

Resistance training led to decreased expression of myostatin in 73% in active muscles [101]. The reduction in myostatin expression was observed in a single session and after 9 week of traditional high-intensity (i.e., 75%-85% one-repetition maximum) resistance exercise (34%) [98,101]. These findings suggest that downregulation of myostatin gene after exercise may result in greater muscle hypertrophy in a training program. Accordingly, Laurentino et al. [102] showed that increased in muscle strength and hypertrophy responses observed after either low-intensity resistance exercise associated with moderate blood flow restriction or high-intensity resistance exercise were related with similar changes in selected myostatin-related genes mRNA expression. The authors found reduction in myostatin gene and a trend in Activin IIb mRNA expression after 8 weeks of training in both the protocols. Interestingly, the study also report significant increases in GASP-1 and Smad-7 gene expression after exercise training. Furthermore, studies show that in elderly who underwent strength training was observed decreased expression of myostatin in 48% after the last training session only in trained subjects, however, was observed desensitization of the receptor Activin IIb, even after a single exercise session [98] (Figure 2). However, reduction in myostatin expression induced by exercise is still controversial. In rats, it was observed increased expression of myostatin after 30 minutes in a single session of eccentric exercise [103]. Studies show that resistance training increased muscle expression of myostatin and its circulating levels [104]. It may be speculated that these dissonant findings are related to the timing of the biopsy after the last training session, once that studies performed posttraining biopsies 48-72 h after the last training session, whereas others collected the samples only 15 min after it. Therefore, it is possible that these findings may reflect acute and training program effects, respectively.

Studies on the expression of myostatin-related loss of muscle mass promoted by physical detraining were developed. Jespersen et al. [105] performed muscle biopsies obtained from young male subjects before and after 30 and 90 days of resistance training as well as after 3, 10, 30, 60 and 90 days of subsequent detraining. Myostatin mRNA increased significantly with detraining. Further, they observed a significant increase in this expression after 3 days of detraining preceding the rapid type II fiber atrophy, in which almost half of the acquired fiber area was lost after only 10 days of detraining. Thus, the data suggest a role for myostatin in the negative regulation of adult human skeletal muscle mass.

In contrast to the canonical view of skeletal muscle structure and function, the muscle hypertrophy that develops in the absence of myostatin is not accompanied by proportionate increase in contraction strength [106], however, recent evidence suggested that endurance exercise training may normalize the muscle phenotype induced by the absence of myostatin [107,108]. Matsakas et al. [108] showed that two different types of endurance training, voluntary wheel running and swimming reduced muscle fiber size, increased muscle oxidative properties, increased capillary density and, most importantly, improved force generation in the myostatin null mouse. Thus, these results demonstrate that features

induced by a germ-line deletion of myostatin are not genetically locked down but can be modified by exercise training (Figure 2).

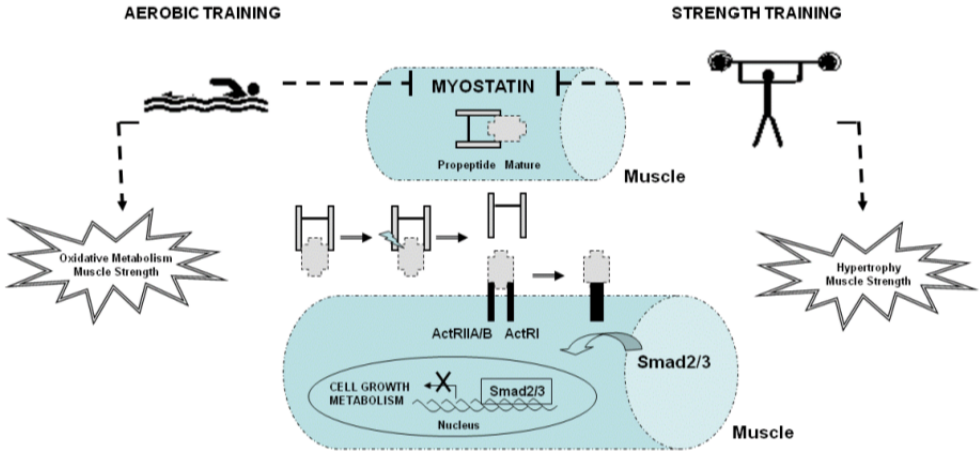

Figure 2. Effect of exercise training on skeletal muscle myostatin expression. Inactive myostatin (propeptide portion forms non-covalent link to mature portion) is secreted by muscle cells. Proteases on muscle cells release propeptide from mature region. Mature region binds type II Activin receptor. Transphosphorylation leads to activation of type I receptor which it phosphorilates Smad2/3 facilitating the translocation into nucleus where it initiates genes transcription related to cell growth and metabolic change toward glycolitic profile. Interestingly, aerobic and strength training reduce skeletal muscle myostatin expression associated to increase muscle strength, hypertrophy and metabolic homeostasis

Myostatin may be related pathways that contribute to muscle regeneration after exercise. Consequently, these changes will depend on the conditions myostatin muscle before exercise. Studies comparing people with different types of training prior show that the response of myostatin can be altered with resistance exercise [109]. This hypothesis is strengthened by the fact that myostatin expression is increased in response to elevated serum levels of glucocorticoids. The regulatory region of the myostatin gene contains sequences activating responsive to glucocorticoids [110]. Thus, the increase in protein may be due to stress caused by exercise training [104]. However, studies have shown that increased expression of myostatin induced by exercise occurred concomitantly with increasing of FLRG and decreasing Activin receptor IIb levels, suggesting that the increase in FLRG can inhibit the myostatin activity in those cases, occurring mechanism compensatory increase the myostatin [88].

In cardiovascular diseases as late-stage chronic heart failure and diabetes, elevated cytokines and cachexia are often observed. Several studies have shown that exercise training exerts beneficial effects on skeletal muscle in this setting. Furthermore, it has been shown that the expression of myostatin is increased in a variety of cachectic states. Myostatin is capable of inducing muscle atrophy via its inhibition of myoblast proliferation, increasing ubiquitin-proteasomal activity and downregulating activity of the IGF–Akt pathway [111-113].

Remarkably, exercise training on a treadmill over 4 weeks led to a significant reduction in myostatin protein expression in the skeletal muscle and the myocardium of chronic heart failure animals, with values returning to baseline levels [111]. In addition, chronic heart failure patients showed a two-fold increase of myostatin mRNA and a 1.7-fold augmentation of protein content in skeletal muscle compared to healthy subjects. However, exercise training led to a 36% reduction of the mRNA and a 23% decrease of the myostatin protein compared to baseline [112]. Accordingly, myostatin, its receptors and follistatin expression change in both muscle and fat of diabetic rats and their expressions can be modulated by exercise in diabetes [113]. These alterations in myostatin expression in the skeletal muscle following exercise training could help to explain the beneficial anti-catabolic effects of exercise training in cardiovascular diseases.

In conclusion, this approach appears to have important inhibitory role for the hypertrophy induced by both aerobic and resistance exercise by being used as a negative regulator of hypertrophy. However, we need more studies to establish a direct relationship between this protein and hypertrophy induced by exercise and thus clarify the role of changes in their expression after a workout, both in aerobic and resistance exercises.

5. Skeletal muscle and microRNAs

Skeletal muscle cells arise from embryonic mesoderm during embryonic development, where they exist as proliferating myoblasts or terminally differentiated myotubes that have exited the cell cycle [114]. Recent studies have revealed that, in addition to activating genes involved in muscle differentiation and muscle contraction, these myogenic transcription factors activate the expression of a set of conserved microRNAs (miRNAs) that function to "fine-tune" the output of these transcriptional networks, resulting in accurate cellular responses to developmental, physiologic and pathologic signals [114-116].

MiRNAs are a class of short, non-coding RNA molecules that reportedly play a central role in regulating post-transcriptional gene expression during embryonic stem cell development, myogenesis, adipogenesis, fat metabolism and glucose homeostasis [116]. MiRNAs are ~22 nucleotides long and inhibit translation or promote mRNA degradation by annealing to complementary sequences in the 3′untranslated regions (UTRs) of specific target mRNAs. It is estimated that there are more than 1500 miRNAs encoded by the human genome, roughly equaling the number of transcription factors [117]. The power of miRNAs as regulators of gene expression is also underscored by recent study demonstrating their ability to up-regulate translation of specific targets [118].

Approximately 50% of mammalian miRNAs loci are found in close proximity to other miRNAs. These clustered miRNAs are transcribed from a single polycistronic transcription unit (TU), although there may be exceptional cases in which individual miRNAs are derived from separate gene promoters. Some miRNAs are generated from non-coding TUs, whereas others are encoded in protein-coding TUs. Approximately 40% of miRNAs loci are located in the intronic region of non-coding transcripts, whereas ~10% are placed in the exonic

region non-coding TUs. MiRNAs in protein-coding TUs are usually found in intronic regions. Some mixed miRNA genes can be assigned to either intronic or exonic miRNA groups depending on the alternative splicing patterns [119].

Most miRNAs are transcribed by DNA-dependent RNA polymerase II (RNAPII) to generate a primary miRNA (pri-miRNAs) is processed in the nucleus by the RNase Drosha, yielding stem-loop structures of ~70 nucleotides. These precursor (pre-miRNAs) are transported to the cytoplasm by the nuclear export protein, Exportin 5, where they are further processed by the RNAse Dicer, giving rise to the mature miRNA and its complementary strand from the stem-loop, referred to as the ´star` strand [5]. The mature miRNA is incorporated into the RNA-induced silencing complex (RISC), where acts by hybridizing either perfectly or partially to complementary binding sites located in the 3`UTRs of target mRNAs, promoting translational repression or degradation [115,119] (Figure 3).

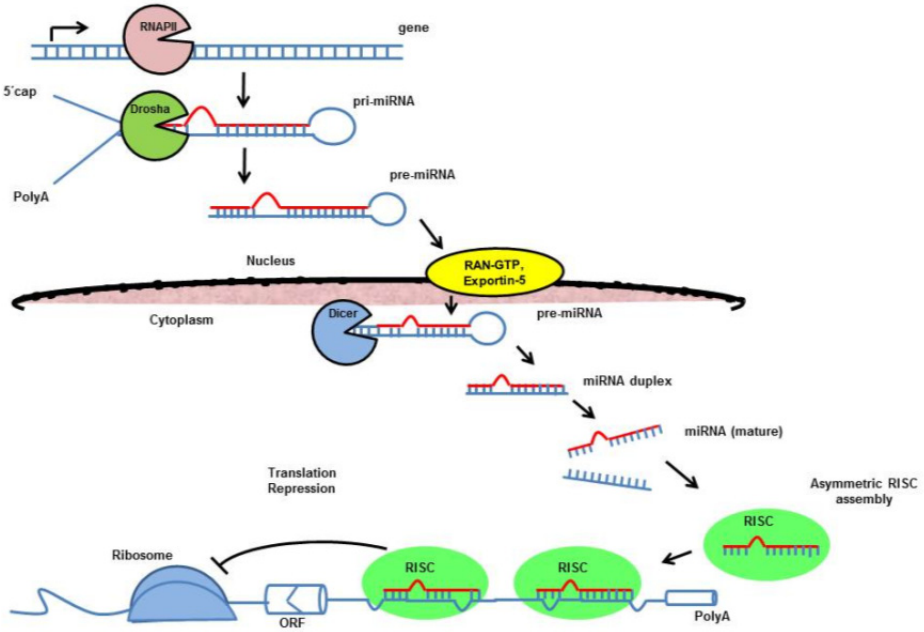

Figure 3. The Current model for the biogenesis and post-transcriptional supresssion of microRNAs – MicroRNAs are generally transcribed by RNA polymerase II (RNAPII) to yield primary miRNAs (pri-miRNA) transcripts are first processed into ~70 nucleotide pre-miRNAs by Drosha inside nucleus. Pre-miRNAs are transported to the cytoplasm by Exportin 5 and are processed into miRNAs by Dicer. Only one strand of the miRNA duplex is preferentially assembled into the RNA-induced silencing complex (RISC), which subsequently acts on its target by translational repression. ORF, open reading frame.

Recent evidence supports a role for miRNAs as integral components of the regulatory circuitry for muscle development [120]. In skeletal muscle, miRNA-1, miRNA-133a, miRNA-133b, and miRNA-206 together account for nearly 25% of all miRNA expression and are as a

group often referred to as myomiRNAs [121]. The expression of myomiRNAs is dramatically increased during myogenesis. Furthermore, differential expression of myomiRNAs following resistance exercise in skeletal muscle suggests that myomiRNAs play a role in human health [115].

The functional characterization of miRNA-1, miRNA-133a, miRNA-133b and miRNA-206, has been an important step in our understanding of miRNA-mediated muscle development.

Studies have demonstrated that miRNA-1 and miRNA-133 regulate fundamental aspects of muscle biology such as differentiation and proliferation. In C2C12 skeletal muscle cells, miRNA-1 represses the expression of histone deacetylase 4 (HDAC4), a negative regulator of differentiation and a repressor of the MEF2 (transcription factor). Thus, the repression of HDAC4 by miRNA-1 establishes a positive feed-forward loop in which the up-regulation of miRNA-1 by MEF2 causes further repression of HDAC4 and increased activity of MEF2, which drives myocyte differentiation [116]. In C2C12 myoblasts, the ability of miRNA-133 to promote proliferation has been ascribed to the repression of SRF (serum response factor), an essential regulator of muscle differentiation. MiRNA-133 also represses translation of the polypyrimidine tract-binding protein (nPTB), which promotes differential splicing of a variety of transcripts that influence the muscle differentiation program [122]. In addition, CHIP on CHIP analysis also indicated that the myogenic regulatory factors, MYOD1 and Myogenin, bind to sequences upstream of miRNA-1 and miRNA-133. It seems as miRNA-1 and miRNA-133 that are encoded by the same MEF2-regulated bicistronic transcripts would exert opposing effects on muscle growth and differentiation. However, both miRNA-1 and miRNA-133 fine tune key regulatory pathways in an antagonistic manner with the balance being tipped one way or the other by additional transcription factors and regulatory pathways. While experiments in cell culture suggested that miRNA-1 and miRNA-206 promote differentiation of myoblasts, miRNA-133 has been proposed to promote myoblast proliferation, a role opposite to that of miRNA-1 through down-regulation of different target gene [120].

Most recently, miRNA-1 and miRNA-133 were shown to play regulatory a role in apoptosis. MiRNA-1 mediated a pro-apoptotic effect, while the effect of miRNA-133 was anti-apoptotic [123]. Thus, in addition to their role in regulating muscle cell proliferation and differentiation, miRNA-1 and miRNA-133 also seem to play opposing roles in regulating muscle cell apoptosis. The opposing effects of miRNA-1 and miRNA-133 during apoptosis are likely explained by which genes are targeted: miRNA-1 reduced protein levels of HSP60 and HSP70, while miRNA-133 repressed caspase-9 expression [123]. Though a clear picture of which genes are regulated by miRNAs is desperately needed to fully understand the roles of miRNAs in muscle biology, the main theme that has emerged thus far is that miRNAs indeed participate in regulatory networks modulate muscle gene expression, muscle cell proliferation, differentiation, and apoptosis [124, 125].

MiRNA-206 is one the most abundant miRNAs in adult skeletal muscle. The function of miRNA-206 in adult skeletal muscle remains to be determined but given the importance of the muscle-specific miRNAs in muscle development, it is reasonable to propose an

important role for myomiRNAs in skeletal muscle plasticity in the adult animal. Recent study determined the expression of precursor and mature forms of the established myomiRNAs in the soleus and plantaris muscles and in the plantaris during the initial stage of muscle hypertrophy. Interestingly, expression of miRNA-206 was 7-fold higher in the soleus muscle in comparison to the plantaris muscle suggesting miRNA-206 may have some role in a specifically setting fiber type. During skeletal muscle hypertrophy induced by synergist ablation, transcript level of precursor miRNA-206 (pri-miRNA-206) was elevated 18.3 fold whereas expression of miRNA-206 did not significantly change. The reason for this post-transcriptional regulation of miRNA-206 during muscle hypertrophy is not known but the authors suggested it might be the result of competitive inhibition of Drosha by ribosomal RNA (rRNA). Whatever the reason for the discordant expression of pri-miRNA-206 and miRNA-206 during hypertrophy, it would be of interest to determine if at a later time point, when the fast-to-slow fiber type transition is known to occur, if there is an increase miRNA-206 expression comparable to pri-miRNA-206 levels. If this scenario was found to be the true, it would provide further evidence to support the idea that miRNA-206 is involved in regulating fiber types [126,127].

The miRNA-206 has been characterized as a muscle regulator and has also been shown to promote myoblast differentiation. Gap junction protein connexin43 (Cx43) and the p180 subunit (Pola1) of DNA polymerase alpha have been identified as regulatory targets of miRNA-206. Although Cx43 is required for the initial phase of myogenesis, it is rapidly down-regulated post-transcriptionally after the induction of differentiation, thus miRNA-206 is suggested to decreased communication between development muscle fibers decreasing Cx43 expression [128-130]. Down-regulation of Pola1 by miRNA-206 during early differentiation reduces DNA synthesis and contributes to the suppression of cell proliferation during myotube formation. MiRNA-206 is also suggested to mediate MyoD-dependent inhibition of follistatin-like 1 (FSTL1) and Utrophin (Utrn) genes in myoblasts. In this case, MYOD1 activates the expression of miRNA- 206, which in turn represses FSTL1 and Utrn gene expression post-transcriptionally. This mechanism could explain some of the previous observations in which MYOD1, known as a transcriptional activator, repressed FSTL1 and Utrn gene expression. Although Utrn expression was repressed by miRNA-206 during myoblast differentiation, its expression was up-regulated in *mdx* diaphragm muscle. This phenomenon might reflect decreased efficiency of miRNA-mediated translational repression during a diseased state [120,130,131].

Interestingly, recent studies show that miRNA-214 is expressed in skeletal muscle cell progenitors during zebrafish development and was shown to specify muscle cell type during somitogeneses by modulating the response of muscle progenitors to Hedgehog signaling. Blocking miRNA-214 activity by injecting chemically-modified antisense oligonucleotides into zebrafish embryos decreased in the number of slow-muscle cell types present in the developing somites and distinctly changed the gross morphology of the somites in manner previously associated with attenuated Hedgehog signaling. This phenotype was attributed to relief of miRNA-214-mediated inhibition of *suppressor of fused (su(fu))* expression , a fine tuner of Hedgehog signaling essential for proper specification of

muscle cell types during somitogeneses [120,132]. It will be interesting to test whether miRNA-214 plays a similar role in mammalian skeletal muscle development. Collectively, these studies indicate that miRNAs function as regulators of gene expression important for myoblast proliferation and differentiation and may play decisive roles in specifying cell types during development [120].

In contrast to the other muscle miRNAs discussed, which are specifically expressed in a tissue-restricted manner, miRNA-181 is broadly expressed. Interestingly, the expression of miRNA-181 was increased in the regenerating muscle from an *in vivo* mouse model of muscle injury [133]. Further analysis using the C2C12 cell line demonstrated that miRNA-181 depletion reduced MyoD expression and inhibited myoblast differentiation. One of the genes targeted by miRNA-181 is homeobox protein Hox-A11, which in turn represses MyoD expression. The proposed mechanism underlying miRNA-181 function is that miRNA-181 becomes up-regulated upon differentiation and targets a repressor (Hox-A11) of the differentiation process to allow new muscle growth. This study suggests that miRNAs can play roles in establishing a differentiated phenotype and alludes to the potential role of miRNAs in skeletal muscle regeneration. In addition to myogenesis, miRNA-181 was shown to modulate hematopoietic lineage differentiation in another study [134], which suggests that individual miRNAs may play very diverse biological roles depending upon their cellular context [120].

Non-muscle specific miRNAs that contribute to the maturation of myoblasts is miRNA-29, which functions as an enhancer of skeletal myogenesis [135]. In the attempt to identify other miRNAs involved in muscle development, Huang and co-workers performed a global miRNAs expression analysis from porcine skeletal muscle collected in 33-day and 65-day post-gestation fetuses, as well as in adult tissue. Their results revealed that the miRNA-29 family members are strongly regulated during development as the highest expression of all three miRNA-29 members occurred in adult muscle tissue [136]. Additional miRNAs array analysis from a broad spectrum of muscular dystrophies affirmed the expression of miRNA-29 in skeletal muscle and further described the associated reduction of this miRNA in multiple muscle disorders that included Duchenne muscular dystrophy, facioscapulohumeral muscular dystrophy, and nemaline myopathy [137,138].

Our understanding of miRNAs biology is still incipient. It has been estimated that at least one this mammalian genes are regulated by as many as a thousand miRNAs, only a few of which have been studied in any detail. An important challenge for the future will be to identify the downstream targets that mediate the actions of miRNAs in development. The ability of mutations or single nucleotide polymorphisms to destroy, alter or create new target sequences for miRNAs represents an intriguing source of phenotypic variation. Such polymorphisms will likely be difficult to identify, given the degeneracy within miRNA-mRNA interactions and relatively short sequences of miRNAs and their targets. Finally, although muscle has been among the most intensely studied cell type with respect to the regulation and mechanisms of action of miRNAs, the principles learned from muscle will undoubtedly apply to other cell types.

6. Conclusion

Considerable progress has been made in understanding the signaling pathways that mediate the hypertrophy and atrophy of skeletal muscle. The present literature supports the role of activation of signaling pathways intracellular Akt- mTOR, myostatin and skeletal muscle miRNAs in regulating hypertrophic by increasing muscle protein synthesis induced by exercise training. However, the mechanisms that regulate this process are quite complex and sometimes controversial in the literature, requiring greater effort and future studies to further elucidation.

As already mentioned, the objective of this review was to identify and discuss the main factors in the literature as capable of generating the hypertrophic response, ie the various intracellular signaling pathways that produce the biochemical responses promoters of increasing muscle fiber size. Certainly, there are other avenues to be considered, but these identified here may be regarded as the most studied and best representing the complex signaling system responsible for the intracellular skeletal muscle trophism induced by exercise training.

Regimes that can stimulate muscle growth and prevent muscle loss are likely to benefit a significant proportion of the population. These findings may greatly contribute to the importance of exercise training in future interventions pharmacological and clinical, especially for prevention and control of diseases, as well as for future insertions sports performance, rehabilitation and aging.

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Chapter 10

Mitochondrial Biogenesis in Skeletal Muscle: Exercise and Aging

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Additional information is available at the end of the chapter

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1. Introduction

Mitochondria are abundantly present in mammalian cells. Their fraction varies from tissue to tissue, ranging from <1% (volume) in white blood cells to 35% in heart muscle cells. However, mitochondria should not be thought of as single entities, but rather a dynamic network that continuously undergoes fission and fusion processes. In skeletal muscle, mitochondria exist as a reticular membrane network. The subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria are located in distinct subcellular regions, and they possess subtle differences in biochemical and functional properties that are characterized by their anatomical locations. SS mitochondria lie directly beneath the sarcolemmal membrane and the IMF mitochondria are located in close contact with the myofibril. Their different properties are likely to influence their capacity for adaptation. SS mitochondria account for 10-15% of the mitochondrial volume and this population has been shown to be more susceptible to adaptation than the IMF mitochondria. However, the IMF mitochondria were found to have higher rates of protein synthesises, enzyme activities and respiration (1).

The mitochondria are equipped with double membranes, crating the intermembrane space between the outer and inner membranes as well as the inner matrix compartment, where most of the metabolic processes take place. The inner membrane is highly folded, forming so-called cristate, to accommodate its large surface area. Embedded in the inner mitochondria membrane are the five complexes that make up the respiratory chain where oxidative phosphorylation takes place. In this process, a proton gradient across the inner membrane is coupled to ATP synthesis at complex V (2). In addition to producing ATP essential for cell survival, the mitochondria are a source for free radical or reactive oxygen species (ROS), production. ROS are small, highly reactive molecules that can be generated by mitochondrial respiration and in active skeletal muscle.

Mitochondria are unique organelles in that they contain their own DNA, which consists of a circular DNA molecule of about 16.6 kb in humans and 16.3 in mice. It encodes 13 of the

around 90 proteins that make up the respiratory chain. In addition, mtDNA also encodes 2 ribosomal RNAs (rRNA) and 22 transfer RNAs (tRNA) (3). The presence of mtDNA is explained by the evolutionary origin of mitochondrion as a free-living prokaryotic organism. During the course of time, genes have been transferred to the nuclear genome, and mitochondrial function is highly depended on close coordination between the nuclear and mitochondrial genomes. In mammals, mtDNA is maternally inherited, the paternal mtDNA being destroyed during the first embryonic cell divisions. The individual stands of mtDNA are termed heavy (H) and light (L) stand. Introns are lacking, but there is a long non-coding region, the D loop, which contains control elements for transcription and replication of mtDNA.

The mitochondria are often referred to as the powerhouses of the cell. In turn, It is well stabilised that mitochondria are the site of oxidative energy production in eukaryotic cells and provide the majority of the total ATP required to maintain normal cellular function and homeostasis. Within skeletal muscle, ATP is primarily required for the energy-dependent cross-bridge cycling between actin and myosin, as well as for Ca^{2+} cycling. Within the mitochondrial matrix, enzymes oxidize fatty acids and carbohydrates producing the reducing equivalents, NADH and FADH2. These reducing equivalents are then used to produce a proton gradient across the inner mitochondrial membrane. Dissipation of this gradient through the F0F1-ATPase results in the resynthesis of the ATP that drives every energy-dependent process in the cell. Studies showed Changes in metabolic demand can directly alter the concentration of mitochondria within the cell. Proliferation of mitochondria occurs in muscle in response to endurance exercise training, chronic electrical stimulation and thyroid hormone, while loss of mitochondria is associated with inactivity and aging.

2. Mitochondrial biogenesis – Effects of exercise

Skeletal muscle is a highly malleable tissue, capable of considerable metabolic and morphological adaptations in response to repeated bouts of contractile activity (i.e. exercise). It is well established that chronic contractile activity, in the form of repeated bouts of endurance exercise, usually interspersed with recovery periods, results in the altered expression of a wide variety of gene products, leading to an altered muscle phenotype with improved fatigue resistance. This improved endurance is highly correlated with the increase in muscle mitochondrial density and enzyme activity, referred to as 'mitochondrial biogenesis'. Mitochondrial biogenesis within muscle consists of two possible mutually inclusive alterations: [1] an increase in mitochondrial content per gram of tissue and/or [2] a change in mitochondrial composition, with an alteration in mitochondrial protein-to-lipid ratio (4). Although this phenomenon resulting from exercise has long been established, many of the detailed molecular mechanisms remain to be identified. This has particular relevance for our understanding of the pathophysiology of mitochondrially based diseases, and may improve our understanding of mitochondrial pathways involved in programmed cell death. Additionally, it has been suggested that an age-related accumulation of dysfunctional mitochondria may result in progressive reactive oxygen species-induced damage, producing a further impairment of oxidative capacity in aged muscle. Moreover,

dysfunctional mitochondria have also been implicated in the age-related loss of muscle mass known as sarcopenia. Thus, mitochondrial biogenesis induced by chronic exercise is now recognized to have implications for a broader range of health issues than just the enhancement of endurance performance.

Therefore, the present chapter will highlight important molecular mechanisms that involved in mitochondrial biogenesis and then we will investigate the exercise effects on theses mechanisms. In the second Section of theses chapter, we examine the effects of aging on mitochondrial content and function and potential role of exercise in attenuation of age-related mitochondrial dysfunction.

2.1. Most important mechanism that involved in mitochondrial biogenesis

2.1.1. Mitochondrial biogenesis requers the corporation of the nuclear and mitochondrial genomes

One of the most fascinating aspects of mitochondrial synthesis is that it requires the cooperation of the nuclear and mitochondrial genomes (Figure-1). Mitochondria are unique in the fact that they house multiple copies of a small circular DNA molecule (mtDNA) comprising 16,659 nucleotides. As noted above, this mtDNA is minuscule compared with the 3 billion nucleotides found in the nuclear genome, it nonetheless contributes 13 mRNA, 22 tRNA, and 2 rRNA molecules that are essential for mitochondrial function. The thirteen mRNA molecules all encode protein components of the respiratory chain, responsible for electron transport and ATP synthesis.

Where does the cooperation between the genomes come in? First, these thirteen components comprise only a small fraction of the total respiratory chain proteins. Some act as single protein subunits, but many are combined nuclear-encoded proteins to form multisubunit holoenzymes, like COX or NADH dehydrogenase (Figure-1). The function of these holoenzymes is clearly impaired if contributions from either genome is absent (5). Second, it is known that mtDNA transcription and replication require the import of nuclear gene products, which act as polymerases or transcription factors. Given the diverse promoter regions of nuclear genes encoding mitochondrial proteins, as well as the sequences of the mtDNA promoters, it is not surprising that this coordination can be disrupted. Evidence for this has been presented in cases of thyroid hormone treatment, suggesting that a coordination of gene expression responses leading to strict stoichiometric relationships is not absolutely necessary for the formation of a functional organelle (6).

2.1.2. Exercise effects on corporation of the nuclear and mitochondrial genomes

A longstanding question has been related to how the two genomes are regulated, or coordinated, in response to a stimulus leading to mitochondrial biogenesis. Williams et al. (7-8) were the first to show that chronic contractile activity led to increases in mRNA levels encoding both nuclear and mitochondrial gene products. Subsequently, this was demonstrated for subunit mRNAs belonging to the same COX holoenzyme. Because COX

contains 10 nuclear encoded and 3 mitochondrial-encoded subunits, this enzyme is a useful model for studying the interactions of the two genomes. The mRNA expression of these subunits is also coordinated across a variety of tissues possessing a wide range of mitochondrial contents. In addition, some evidence for a coordinated regulation of the two genomes was found during the mitochondrial biogenesis induced by cardiac hypertrophy, as well as in human muscle when trained and untrained individuals were compared.

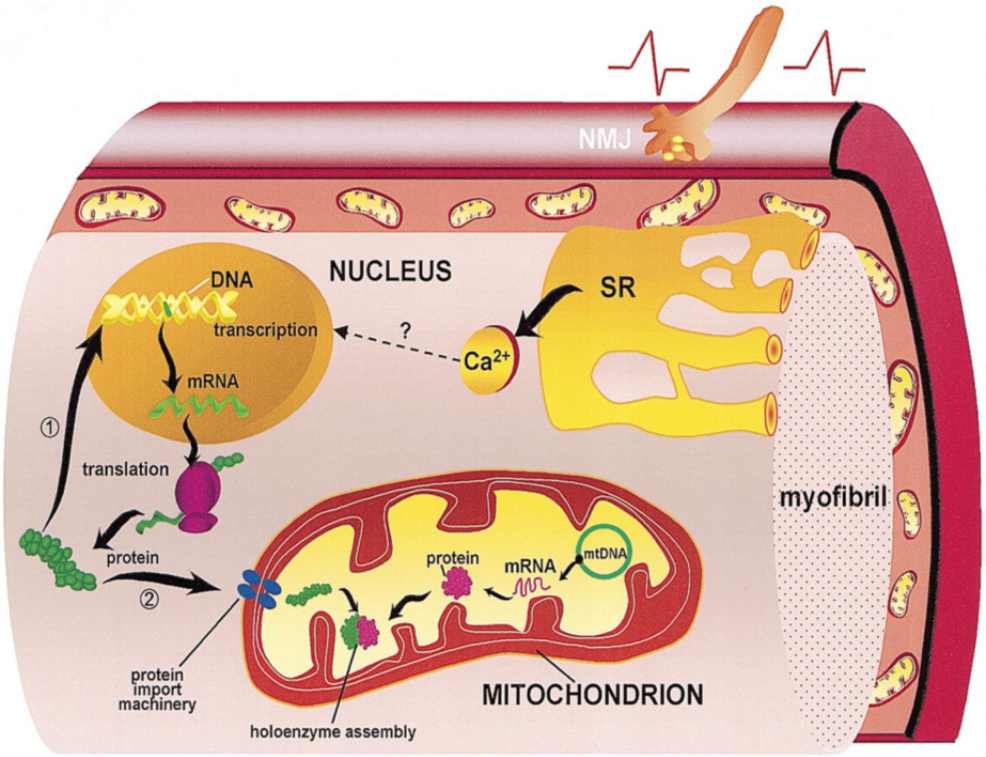

Figure 1. Overall synopsis of mitochondrial biogenesis in a muscle cell. Signals originating at the neuromuscular junction (NMJ) include propagated action potentials and the release of trophic substances, which interact with the postsynaptic membrane. Electrical activity in the sarcolemma is coupled to the release of calcium from the sarcoplasmic reticulum (SR). Calcium acts as a second messenger to activate phosphatases and/or kinases, which are ultimately translocated to the nucleus to affect the activation of transcription factors and which influence the expression of nuclear genes encoding mitochondrial proteins. mRNA produced by transcription is translated into protein in the cytosol, which can either be translocated back to the nucleus (transcription factor) or chaperoned to the protein import machinery and taken up by the organelle. Within mitochondria it may act as a single protein subunit or be combined with other subunits to form a multisubunit holoenzyme (e.g., cytochrome c oxidase). Some subunits of the holoenzyme may be derived from the mitochondrial genome (mtDNA), which also undergoes transcription and translation to synthesize a limited number (13) of proteins that are essential components of the electron transport chain.

2.1.2.1. Protein import machinery (PIM)

The expansion of the mitochondrial reticulum in skeletal muscle is a highly regulated and complex process that appears to require the co-ordinated expression of a large number of genes. Thus, an important aspect of mitochondrial biogenesis is the import machinery regulating the transport of nuclear encoded precursor proteins into the organelle. The vast majority of mitochondrial proteins (>90%) are encoded by nuclear genes and synthesized in the cytosol as preproteins containing a mitochondria import sequence.

Notwithstanding the importance of the mitochondrial genome in contributing proteins to the mitochondrial respiratory chain, it is nevertheless true that most mitochondrial proteins are derived from nuclear DNA. Therefore, a mechanism must exist for targeting these proteins to specific mitochondrial compartments once they have been synthesized in the cytosol. Most proteins are fabricated as "precursor" proteins with a signal sequence, often either located at the NH2 terminus or as an internal sequence (Figure-2).

Although pathways of protein targeting to the outer membrane, inner membrane, matrix, or intermembrane space differ somewhat from each other (9), the most widely studied path is that of proteins destined for the matrix. In this case, the positively charged NH2-terminal signal sequence interacts with a cytosolic molecular chapter that unfolds the precursor and directs it to the outer membrane import receptor complex, termed the translocase of the outer membrane (Tom complex). Cytosolic chaperones include 70-kDa heat shock protein (HSP70) and mitochondrial import stimulating factor (MSF). Precursor proteins can be directed to one of two subcomplexes within the Tom machinery. One of these, consisting of the Tom20 and Tom22 receptors, is the preferential route for HSP70 chaperone precursors.

On the other hand, proteins interacting with MSF are largely directed to the Tom70-Tom37 heterodimer (10). Precursors are then transferred from the Tom receptors to Tom40 and the small Tom proteins 5, 6, and 7, which form an aqueous channel through which the precursor protein passes. Proteins are then sorted to the outer membrane, to the inner membrane, or to the translocase of the inner membrane (Tim), another protein complex that allows movement of precursor proteins to either the matrix or the inner membrane. Those proteins involved in the translocation of the precursor to the matrix are Tim17, Tim23, and Tim44. Tim17 and Tim23 act as integral membrane proteins, spanning the mitochondrial inner membrane and having domains associated with both the matrix and intermembrane space. In a manner similar to the Tom receptor complexes, Tim17 and Tim23 bind the precursor protein, prevent any untimely folding that would inhibit the precursor from translocating into the matrix, and form an aqueous pore through which the precursor can travel. In contrast, Tim44 is a peripheral membrane protein that is secured to the inner face of the inner mitochondrial membrane. Tim44 anchors the matrix chaperone HSP70 (mtHSP70), which acts in a ratchet like manner to pull the precursor into the matrix (Figure-2). Along with these proteins, the inner membrane phospholipid cardiolipin is imperative for protein translocation because it appears to orient the precursor into the correct position for interaction with the Tim44-mtHSP70 complex. The importance of this phospholipid has been shown by studies in which cardiolipin function has been blocked using the drug Adriamycin, resulting in an attenuation of the import of proteins destined for the matrix (11-12).

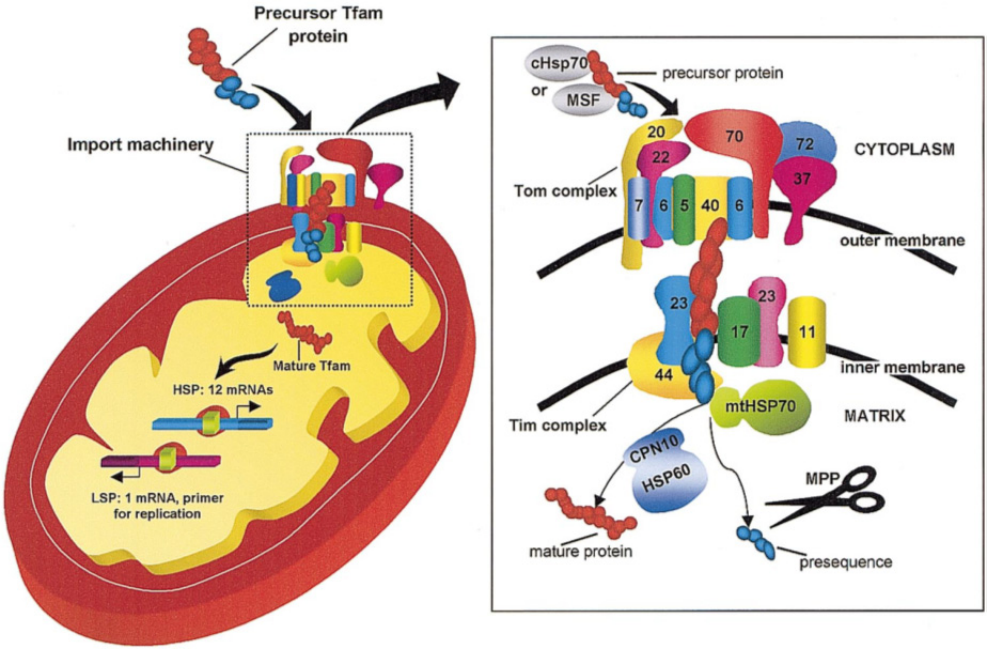

Figure 2. Left: mitochondrial transcription factor A (Tfam) is a nuclear-encoded transcription factor that is synthesized in the cytosol as a larger, "precursor" protein with a positively charged NH2-terminal presequence (blue). It must interact with the protein import machinery to enter the organelle. Once inside the matrix, mature Tfam will bind within the D-loop region of the circular (not shown) mtDNA on the heavy-strand (HSP) and light-strand promoters (LSP) and stimulate the transcription and replication of mtDNA. Right: enlarged view of the components of the protein import machinery. A typical matrix-destined precursor like Tfam is unfolded and directed to the import machinery by a cytosolic chaperone, either cytosolic 70-kDa heat shock protein (cHSP70) or mitochondrial import stimulating factor (MSF). On interaction with the translocase of the outer membrane (Tom complex), it is correctly oriented by interacting with the inner membrane phospholipid cardiolipin (not shown) before being transferred to the translocase of the inner membrane (Tim complex). The matrix chaperone mtHSP70 pulls in the precursor, and the signal sequence is cleaved by the mitochondrial processing peptidase (MPP). Subsequently, the mature protein is refolded by matrix chaperonins HSP60 and Cpn10. ATP is required at multiple steps during the import process. The number within each import machinery component refers to its size in kDa.

Two other elements are required for correct import of precursor proteins into the matrix. These are 1) the presence of an inner membrane potential (DC, negative inside) across the inner membrane to help pull the positively charged presequence into the matrix and 2) the availability of ATP both in the cytosol and in the matrix. Uncoupling agents that dissipate DC reduce protein import, whereas ATP depletion prevents the unfolding of the precursor in the cytosol and/or the action of mtHSP70 in the matrix. Thus reductions in cellular ATP levels such as that produced by severe contractile activity or defects in ATP production as might be encountered in cells with mtDNA mutations could affect the rate of import into mitochondria.

After its arrival in the matrix, the NH2-terminal signal sequence is cleaved by a mitochondrial processing peptidase (MPP) to form the mature protein. It is then refolded into its active conformation by a mitochondrial chaperonin system consisting in part of 60-kDa heat shock protein (HSP60) and 10-kDa chaperonin (Cpn10). The vast majority of work that defines the components of the protein import machinery, as well as their cellular function, has been done in Saccharomyces cerevisiae and Neurospora crassa. This is now being extended to mammalian cells. For example, the kinetics of matrix precursor protein that import into skeletal muscle SS and IMF mitochondrial fractions, the ATP and cardiolipin dependence of the process, and the relationship to mitochondrial respiration have all been defined (13). IMF mitochondria import precursor proteins more rapidly than SS mitochondria, and there is a direct relationship between the capacity for mitochondrial respiration (and thus ATP production) and the rate of protein import. It has also been shown that a number of protein import machinery components are induced in response to chronic contractile activity. These include the chaperones MSF, cytosolic HSP70 (cHSP70), mtHSP70, HSP60, Cpn10, as well as the import receptor Tom20. Coincident with these increases are contractile activity-induced increases in the rate of import into the matrix but not into the outer membrane. This differential effect on protein targeting to mitochondrial compartments provides an example of how contractile activity can result in an altered mitochondrial protein stoichiometry. The accelerated rate of protein import into the matrix can be reproduced in cardiac mitochondria obtained from animals treated with thyroid hormone. Thus the effect is not a unique response to contractile activity but appears to be common to stimuli that increase mitochondrial biogenesis. To more easily define the role of specific components of the import pathway in determining the kinetics of import, measurement of import in intact cells can be employed. When C2C12 cells were incubated with [35S] methionine and the import of radiolabeled MDH into mitochondria was measured, a greater rate of import was found during the progress of mitochondrial biogenesis occurring coincident with muscle differentiation. As expected, thyroid hormone accelerated the rate of import and induced the expression of Tom20. To evaluate the role of Tom20 alone in mediating the accelerated import rate, forced overexpression of Tom20 in these cells using a mammalian expression construct was used. Parallel increases in the rate of import and the magnitude of overexpression were observed. Conversely, inhibition of Tom20 expression using specific antisense oligonucleotides led to equivalent decreases in MDH import. These data suggest that the import of matrix-destined proteins is controlled, at least in part, by the expression of Tom20. The protein import pathway represents an example of intracellular trafficking that is important for organelle biogenesis, and it may, under some conditions, determine the increase in mitochondrial content as a result of chronic exercise. For this to be the case, it must be shown that it is inducible and that it operates at a rate that limits the overall pathway under some conditions (i.e., chronic exercise). If the import rate was slow enough to limit mitochondrial biogenesis, then a pool of precursor proteins in the cell cytosol would be measurable. In the absence of such a pool, the assumption is that newly synthesized precursor proteins are rapidly taken up by mitochondria, and the kinetics does not limit the synthesis of the organelle as a whole. This has yet to be rigorously tested in a cellular system in which any other fates of the precursor (i.e., cytosolic degradation) are blocked. It is possible that the import of proteins might become limiting under conditions

of chronic contractile activity if upstream steps (i.e., transcription, translation) are accelerated such that a saturating abundance of precursor proteins are presented to the import machinery.

In any event, the physiological value of the observed contractile activity-induced increases in mitochondrial protein import is that mitochondria are more sensitive to changes in precursor protein concentration, a situation that would be advantageous for mitochondrial biogenesis at any given upstream production rate of cytosolic precursor proteins. Progress in the area of protein import will advance substantially as additional mammalian homologues of the import machinery are identified. Recently, the first disease that can solely be attributed to a mutation in a protein component of the import machinery has been identified. A mutation in deafness dystonia protein (DPP) results in a neurodegenerative disorder characterized by muscle dystonia, sensorineural deafness, and blindness. DPP has been shown to be a mitochondrial protein that closely resembles Tim8p, a protein of the intermembrane space involved in the import process. In addition, mutations in the import receptor Tom70 have been shown to produce mtDNA rearrangements in the fungus Podospora anserina, presumably because of defective import of a component involved in mtDNA maintenance. The cloning of Tom22, as well as members of the Tim machinery, will be of help in elucidating the functional roles of individual import machinery components in the import process and the relevance of import in mitochondrially based diseases and in organelle biogenesis.

2.1.2.2. Exercise effects on PIM

As noted above, exercise has been shown to induce the expression of several protein import machinery components, occurring coincident with an increased rate of translocation into the mitochondria. In turn, activity-induced changes have been observed in Tom20, Hsp60 and mtHSP70 protein and cpn10 mRNA levels, as well as cytosolic concentrations of Hsp70 and MSF (13-15). Coincident with these changes is acceleration in the rate of protein import into the matrix. Thus, the upregulation of protein import machinery components appears to be an important aspect of mitochondrial biogenesis which occurs with contractile activity. This greater capacity for protein import is physiologically relevant because it means that a greater rate of translocation into the organelle will occur at any given concentration of cytosolic protein produced by translation.

2.1.2.3. Transcription factors that involved in mitochondrial biogenesis

Expression of genes promoting mitochondrial biogenesis is predominantly controlled by the global principles of gene regulation, that is, transcription initiation and interaction at the gene promoter. Therefore, transcription factors and transcriptional co-activators represent critical regulators of mitochondrial biogenesis.

Numerous transcription factors have been implicated in mediating the physiological and metabolic adaptations associated with expression of genes involved in mitochondrial biogenesis. While no single transcription factor has been found to be responsible for the co-ordination of mitochondrial gene expression, several candidates appear to be important for mitochondrial biogenesis. These include two nuclear respiratory factors (NRF-1 and NRF-2), two peroxisome proliferator-activated receptors (PPAR-γ and PPAR-α), specificity protein 1

(Sp1), mitochondrial transcription factor A (Tfam), early growth response gene-1 (Egr-1) and the products of the immediate early genes, c-jun and c-fos. This diversity is important given that the characterization of an assortment of upstream promoter regions of genes encoding mitochondrial proteins has revealed considerable variability in their composition.

NRF-1 and NRF-2 are implicated in the transcriptional control of multiple mitochondrial genes including mitochondrial transcription factor A (Tfam) and identified mitochondrial transcription specificity factors TFB1M and TFB2M, while Egr-1 is associated with promoting transcription of the electron transport chain protein cytochrome C oxidase (COX). The peroxisome proliferator receptor gamma co-activator-1 alpha (PGC-1α) has been established as an important regulator of mitochondria content in skeletal muscle due to its apparent co-activation of multiple mitochondrial transcription factors. Indeed, PGC-1α is the founding member of a family of transcriptional co-activators that has been proposed as a potential "master regulator" of mitochondrial biogenesis (16). In support of this contention, Lin and co-workers (2002) over expressed PGC-1α in mice skeletal muscle and observed increased proportions of type I fibers and increased resistance to fatigue (17). In addition, the biogenesis and maintenance of mitochondrial architecture is controlled by altered rates of mitochondrial protein fusion and fission, a role for which mitofusin (Mfn) 1/2 proteins have been strongly implicated (18).

Similarly, PGC-1α also mediates Tfam activation, a key component in mitochondrial DNA replication and transcription. The NRF-1 transcription factor has been shown to activate Tfam which enhances the capacity for assembly of protein complexes within the mitochondria. Therefore, as a co-activator of NRF-1 transcription PGC-1α is involved in regulating Tfam function. Importantly, Tfam activity appears to increase in response to contractile activity and exercise suggesting enhanced mitochondrial protein assembly with endurance training. Most notably, PGC-1α is the co-activator of the peroxisome proliferator activated receptor (PPAR) family (19). The three PPAR sub-types α, γ and δ have distinct functions but all appear to regulate lipid homeostasis via expression of genes involved in mitochondrial fatty acid oxidation. The initial cellular perturbations associated with the onset of muscle activity leading to the activation of these transcription factors are beginning to be defined (Figure-3).

2.1.2.4. Exercise effects on transcription factors

Researchers showed NRF-1, Tfam and PPAR-γ (has emerged as a potential master regulator of mitochondrial biogenesis) mRNA in response to contractile activity in cell cutlers and endurance exercise in vivo is increased. In turn, studies have been shown that PGC-1α mediates a regulatory pathway involving estrogen-related receptor alpha (ERRα) and Mfn1/2, and this pathway has been shown to be up-regulated following a 10-km cycling time trial (20) . Also, this suggests that a PGC- 1α activated pathway promotes an increase in mitochondrial content in response to endurance exercise through enhanced mitochondrial protein fusion. This provokes an increase in mtDNA transcription and replication. The result is that PGC-1 overexpression can produce an overall increase in cellular oxygen consumption and subsequently, increases the aerobic capacity in endurance activities. The physiological significance of increased PGC-1α-PPAR activated gene expression with

endurance training is an enhanced capacity for fat utilisation during prolonged exercise, and may also be related to fast-to-slow fibre type conversion (21). Indeed, this was highlighted by Wang and colleagues (2004) who generated transgenic mice over expressing PPARδ that resulted in a 2.3-fold increase in mitochondrial DNA content, significant type I fibre transformation and a 90% increase in running performance (22).

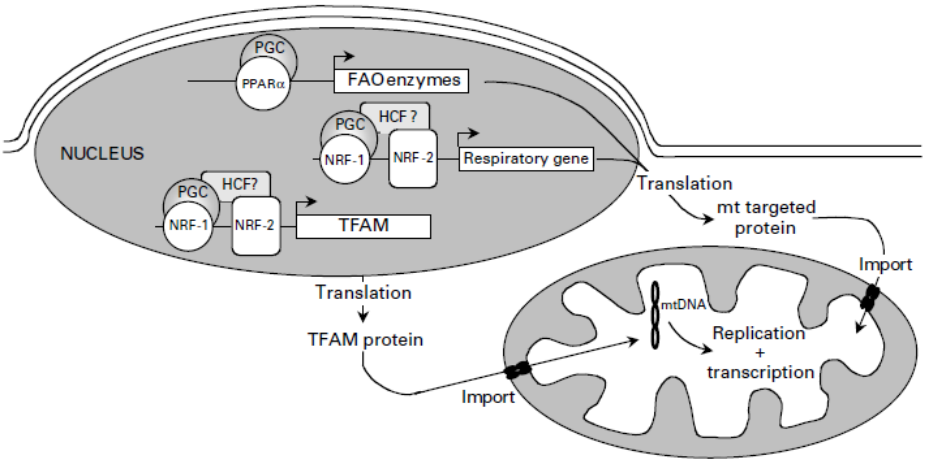

Figure 3. Transcription factors and mitochondrial biogenesis

The small numbers of studies investigating PPAR activation following exercise support these findings where both acute (21, 23-24) and chronic (25-27) endurance exercise induces PPAR transcription. The initial cellular perturbations associated with the onset of muscle activity leading to the activation and increment of these transcription factors are beginning to be defined. A considerable amount of evidence implicates alterations of intracellular Ca^{2+} (28-29) and ATP (30-31) turnover as the initial triggers eliciting the activation of signalling cascades which provoke changes in these gene expressions, as noted above.

3. Mitochondria and aging

3.1. Involment of mitochondria in the aging process

Mitochondria are cited regularly as the main site of superoxide generation that contributes to the majority of reactive oxygen species (ROS) to the cell, although other sites of ROS production within the cell are documented. The potential for ROS to induce oxidative damage has significant implications for the cellular integrity of highly metabolic, long-lived and post-mitotic tissues such as brain, heart, and skeletal muscle. In addition, the effect of ROS is exacerbated by its potential to induce mutations in mtDNA, which is located in close proximity to the source of ROS generation. mtDNA has no protective histones and has substantially less repair mechanisms than nuclear DNA. Thus, ROS-induced accumulations in faulty proteins, oxidized fatty acids, and mtDNA mutations would result in a progressive, feed-forward, and irreversible

cycle of cellular dysfunction that leads to the onset of phenotypes associated with aging. These observations are the major features of the mitochondrial theory of aging, which was first proposed, and then refined, by Denham Harman (32-33), suggesting that changes to mitochondrial integrity, content, and function could have a determining role on the rate at which we age. The role of mitochondria in promoting sarcopenia was uncovered by studies showing that muscle fibers containing dysfunctional mitochondria were atrophied compared to fibers that did not. As well, these authors and other groups (34-36) have reported that histochemical analyses of skeletal muscle fibers revealed an increase in the number of ragged red fibers, characterized by elevated levels of succinate dehydrogenase and a deficiency in COX activity. An in-depth description on the involvement of ROS in mitochondrial dysfunction associated with aging is provided in a later section.

Along with their role in ROS production, mitochondria play a critical role in maintaining cellular integrity through the regulation of programmed cell death, also termed apoptosis. Within mitochondria reside proteins, which upon release from the organelle, can initiate a cascade of proteolytic events that converge onto the nucleus leading to the fragmentation of DNA. This compromises cell viability and ultimately leads to cell death (37). The release of these apoptotic proteins, such as cytochrome C (cytoC), endonuclease G (EndoG) and apoptosisinducing factor (AIF), through either the mitochondrial permeability transition pore (mtPTP) or the homo-oligomeric BAX pores in the outer membrane, occurs in response to cellular stressors such as reactive oxygen species (ROS), chronic elevations in intracellular Ca^{2+} concentration, or gamma irradiation. Thus, the intimate connection between mitochondrial function and the viability of skeletal muscle suggests that this organelle plays a significant role in the progression of aging. Indeed, it is evident that in skeletal muscle of aged individuals, the induction of apoptosis is greater when compared with younger subjects. The increase in cytoC and EndoG release from the mitochondria of aged individuals is paralleled by an increase in caspase-3 cleavage, and p53 mediated apoptosis. The result of apoptosis is a loss in myonuclear number, resulting in a reduction in myofiber diameter to maintain a constant myonuclear domain size. Alternatively, a consequence of fiber atrophy may be the initial activation of apoptotic events that lead to a decrease in myonuclear number. Irrespective of the mechanism involved, mitochondria appear to have an involvement in the progression of sarcopenia. A discussion of the importance of apoptotic signalling during the development of age-related phenotypes caused by mtDNA mutations follows below.

3.2. Alternation in mitochondrial content and morphology with aging

Electron microscopic (EM) analyses reveal that the volume of mitochondria within skeletal muscle declines by 66% with age when compared with younger counterparts (38). Similar EM findings are documented in a human study, revealing a 25% decrease in the density of mitochondria within the vastus lateralis muscle of males and females aged greater than 60 years (39). Related to mitochondrial content is the level of cardiolipin found within skeletal muscle. Cardiolipin is a fatty acid that is exclusively found within the inner membrane of mitochondria, and it is linked to the optimal function and structure of the multitude of enzymes and respiratory complexes. The proximity of cardiolipin to the sites of ROS production makes it

particularly vulnerable to oxidative damage. Numerous studies have investigated whether aging has an effect on cardiolipin content or oxidation in cardiac muscle. Some results have indicated that cardiolipin content is decreased along with an increased degree of peroxidation (40). This is linked to decreased activities of COX, ANT, and carrier complexes. However, other reports have failed to indicate a decline in cardiolipin content or its peroxidation within either SS or IMF mitochondria with age. One study in skeletal muscle has illustrated that cardiolipin content in 36-monthold rats is not decreased when compared with 6-month-old rats in isolated SS and IMF mitochondria (41). However, whether cardiolipin is oxidatively modified with age in skeletal muscle remains to be determined. The morphology of mitochondria may also be altered with age in skeletal muscle, in that a proportion of the organelles are enlarged, depolarized, and non-functional. When compared with the elongated morphology of mitochondria in skeletal muscles of young animals, mitochondria tend to be more rounded in shape within aged skeletal muscle, suggesting that mitochondrial fusion events may be impaired in skeletal muscle. Indeed, decreased OPA1 protein expression has been documented in experimentally-generated, giant mitochondria which may have physiological relevance to the morphology of mitochondria seen in aged individuals (42). Mitochondria have also been shown to undergo significant swelling with age because of the increased retention of calcium. EM has also identified losses in mitochondrial cristae formation, leading to homogenization of the materials found within the mitochondrial Compartments.

3.3. Mitochonrial dysfunction within skeletal muscle of aged individuals

Upstream of the synthesis of ATP, the activities of the metabolic enzymes in Krebs' cycle and those involved in lipid oxidation are altered with age. Citrate synthase activity is significantly decreased with age and the activities of b-hydroxyacyl-CoA dehydrogenase (b-HAD) and succinate dehydrogenase are also reduced with age (43). Oxidation of lipids is also impaired within skeletal muscle of aged individuals. Aged muscle also exhibits characteristics of decreased mitochondrial respiratory capacity and ETC enzyme activities. Functional analyses reveal decreased activities of complex I and IV. In line with these alterations, the activity of COX has been shown to decrease with age and the activities of complexes I, II, III, and IV decrease by 28–43%. Reduced oxidative capacity of approximately 30% has also been reported per mitochondrion (44). As a result of decreased enzyme and complex activities, ATP synthesis and content within aged skeletal muscle is reduced. Thus, there is an increased probability of affecting cellular processes reliant on a constant supply of ATP, such as muscle contractions, protein turnover, and the maintenance of membrane potential.

Skeletal muscle oxidative capacity is a reflection of the ability of working muscle to regenerate ATP through aerobic metabolism. Studies support that whole body maximal oxygen consumption (VO2max) declines with age and there is reduced aerobic capacity per kilogram of muscle in late-middle aged individuals. Oxidative phosphorylation capacity decreased by 50% in 70-year-old human subjects, evaluated using in vivo measurements (39). ATP production rates were decreased by 50% in the gastrocnemius of aged animals (45). Assessments of mitochondrial respiration that was stimulated with a variety of substrates in the presence of ADP revealed that this parameter decreased in aged skeletal

muscle. At rest, muscle ATP synthesis was reduced in 30-month, compared with 7-month-old mice (46). In addition, the ATP content in aged gastrocnemius muscle is 50% lower when compared with that found in young animals (45), and a lower ATP/ADP ratio in 30-month-old mice has been illustrated as well (46).

Despite this evidence, numerous studies have also demonstrated that the oxidative capacity of skeletal muscle does not change with age and discrepancies in results can arise for a number of reasons. One is the lack of consistency of the ages used to make comparisons. Studies may pool together subjects in their late teenage years with middle-aged subjects to represent an ''adult'' group, whereas the ''old'' group could encompass subjects ranging from 40 to 90 years of age. Another variable between aging studies is the differences in the species used, which can range from rats, mice, monkeys, yeast, flies, worms, and humans. The selection of muscle studied, and the method of preparation are also not standardized, such that measurements have been made using either whole muscle homogenates or isolated mitochondrial populations. Related to this, many studies have ignored the potential biochemical differences between the SS and IMF mitochondria and report their findings on mixed mitochondrial samples. It is very possible that these skeletal muscle mitochondrial populations are affected differentially by the aging process. Finally, many studies fail to control for physical activity levels in their subjects, and there is evidence that the majority of age-related declines in mitochondrial oxidative capacity disappear after accounting for this variable (47). Thus, it is controversial whether mitochondrial dysfunction is due to aging per se, or whether the lack of regular physical activity is the major reason for the divergent age-related phenotypes of skeletal muscle. Then again, a reduced oxidative capacity was observed in aged subjects even after accounting for physical activity and fat-free mass. Thus, more research is needed to fully clarify these important issues.

3.4. Causes for the alternations in mitochondrial biogenesis associated with aged skeletal muscle

The impairment in mitochondrial biogenesis may be due to a plethora of causes that lead to the propagation of mitochondrial dysfunction. As discussed below, a change in the content of mitochondria may be due to a decrease in the expression of genes coding for mitochondrial proteins, and/or alterations in the control of protein turnover that occur with aging. In addition, alterations in mitochondrial function may be due to oxidative modifications resulting from an increase of ROS, an elevation of mtDNA mutations, or increased uncoupling of oxidative phosphorylation with age.

3.4.1. Dysregulated experssion of mitochondrial genes

Declines in mitochondrial content and function may be related to the altered expression of nuclear genes encoding mitochondrial proteins (NUGEMPS) in skeletal muscle of the elderly. The huge reliance of mitochondria on the nuclear genome suggests that impaired protein synthesis rates could lead to the decline in mitochondrial biogenesis that is observed with old age, especially if the transcription of NUGEMPS is decreased with age. An

interesting study by Zahn et al. revealed that expression of mitochondrial ETC transcripts decreased, whereas cytosolic ribosomal transcripts were increased in skeletal muscle with age (48). This increased expression of ribosomal subunits may represent a compensatory response for decreased translational efficiency, particularly because protein synthesis has been illustrated to decrease with age. Deficits in ETC enzyme activities have been observed in number of studies and may be linked to a reduction in the transcription of genes located within mtDNA, or to a reduction in the content of mtDNA with age. However, in response to the decline in mitochondrial respiratory function, compensatory increases in mtDNA content in tissues such as skeletal muscle, kidney, and cardiac muscle have been observed. Conversely, the preponderance of evidence seems to suggest that mitochondrial mRNA content is reduced with age. Mitochondrial DNA copy number and mtDNA transcript levels of COX I and COX III have been shown to decrease in 27-month aged animals versus 6-month young animals (49). Similarly in humans, mtDNA content was significantly decreased in muscle biopsies obtained from 67-year-old subjects (50), whereas Welle et al. revealed that mRNA transcripts of components of the respiratory complexes also decrease in their abundance in aged skeletal muscle (51). It has been illustrated that in skeletal muscle of aged humans the rate of mitochondrial protein synthesis is decreased and this may have contributed to the decrease in COX and CS activities observed.

3.4.2. Impaired regulation of protein degradtion

Mitochondrial function and morphology depend on the balance between protein synthesis and assembly, and the clearance of damaged or improperly assembled proteins. A reduced ability of degradation pathways to remove whole or damaged compartments of mitochondria could lead to impaired organelle bioenergetics. These effects likely manifest as decreased ATP synthesis, increased ROS generation, accumulated mtDNA mutations and cell death, characteristics which are observed in skeletal muscle of aging individuals. The major pathways that contribute to mitochondrial protein quality control include intramitochondrial proteases and autophagy. Studies have illustrated that with increasing age, the activity and expression of the intramitochondrial Lon protease is reduced, reflected by an accumulation of dysfunctional aconitase (52). Decreased activity of the Lon protease is likely due to oxidative modifications by elevated ROS levels within the mitochondrial matrix. In the cytosolic environment, lipofuscin has been implicated in contributing to the progressive decline in mitochondrial protein turnover and the onset of dysfunction that occurs with age. Lipofuscin, referred to as the aging pigment, is a non-degradable protein that is the product of incomplete autophagic degradation followed by the peroxidation of remaining contents within the lysosome by reactive oxygen species. Lipofuscin localizes within vesicles throughout tissues in aged individuals, which may reduce the availability of vesicles to form autophagosomes to remove damaged and dysfunctional mitochondria (53). Thus, it appears that the activities of these housekeeping pathways related to protein quality control are altered with aging, resulting in the accumulation of damaged mitochondria and cellular dysfunction. More research is required in this area with skeletal muscle as a function of age.

3.4.3. Elevated damege to macromolecules by ROS

Research unequivocally indicates that ROS production increases in aging skeletal muscle (54). Chabi et al. observed that the generation of ROS is elevated in both the SS and IMF mitochondrial pools of fast-twitch muscles isolated from senescent animals (41). One consequence of increased aberrant ROS production is oxidative damage to complex V leading to a decrease in ATP synthesis and content within skeletal muscle. Additionally, increases in oxidative modifications in DNA occur with age, reflected by higher levels of 8-oxodeoxyguanosine, (8-oxoG) and the corresponding repair enzyme, 8-oxoguanine-DNA glycosylase 1 (OGG1) in skeletal muscle. Increased levels of protein carbonyls have also been associated with aging skeletal muscle. It is well known that slower respiration rates increase the likelihood of the donation of electrons to oxygen at complexes I or III (55), and this may be a feature of mitochondrial respiration in aged individuals. It has also been hypothesized that during aging, there is increased dysfunction of these two complexes, leading to increased ROS generation.

Antioxidant enzymes have evolved to buffer the deleterious, effects of ROS. Enzymes such as manganese superoxide dismutase (MnSOD), catalase (CAT), and glutathione peroxidise (GPX), can ultimately reduce ROS to hydrogen peroxide (H2O2), and then finally into water. The role of ROS in limiting lifespan was elucidated in an elegant study, in which human CAT was targeted to the mitochondria. This resulted in improved aconitase activity, decreased mtDNA mutations, and increased mean lifespan (56). Conversely, transgenic mice lacking copper/zinc SOD (CuZnSOD) showed rapid aging and muscle atrophy similar to sarcopenia in concert with elevated oxidative modifications in proteins, lipids, and DNA, when compared with wild-type animals (57). However, whether the activity or content of these antioxidant enzymes is truly altered with age remains an equivocal issue. A number of studies have illustrated that there is an increase in antioxidant activities with age, as this would be the intuitive hypothesis in response to the elevated ROS generation that occurs during aging (58-59). However, other studies suggest that CuZnSOD, MnSOD, CAT, and GPX activities decrease with age in skeletal muscle, even though the protein and mRNA content of these enzymes were either unchanged or decreased with age (60-61). To add more complexity to this issue, it remains to be determined whether antioxidant enzyme activities are differentially affected by age in the two mitochondrial subfractions. One study suggests that there is no change in the content of MnSOD in SS and IMF mitochondria from tibialis anterior and extensor digitorum longus muscles of aged, compared with young animals. In cardiac muscle, IMF mitochondria exhibit increased levels of GPX, CAT, and MnSOD with age, whereas SS mitochondria exhibit increased levels of GPX and MnSOD and a decrease in CAT activity (62). Because it is clear that oxidative modifications to mitochondrial macromolecules are indeed occurring in skeletal muscle with age, it is likely that the increased ROS production overwhelms the buffering capacity of the antioxidant enzymes that are available. This suggests that other means to reduce ROS, independent of antioxidant enzyme activities, would be beneficial in reducing cellular oxidative damage.

3.4.4. Elevated mutation in mtDNA

An important component of the free radical theory of aging is that mitochondrial dysfunction is a result of accumulated oxidative damage to mtDNA, leading to mutations in coding regions for ETC proteins. The last point is especially critical because mtDNA contains no introns or spacer regions (63), thus even point mutations could lead to the expression of faulty proteins. It is accepted that ROS generation by skeletal muscle mitochondria increases with age and is accompanied by an increase in mtDNA mutations, impaired energy production, mitochondrial dysfunction, and a greater susceptibility to undergo apoptotic signalling that results in the downfall of skeletal muscle function. The most common mtDNA mutation associated with aging has a frequency rate of 30–35%, is found within the D-loop region, and is a deletion mutation that affects the expression of seven of the 13 proteins encoded by mtDNA (64-65). In addition, mtDNA deletion mutations appear to be highly localized in small regions of muscle fibers in mosaic patterns, rather than distributed ubiquitously throughout aged skeletal muscle.

Interestingly, research has illustrated that mtDNA mutations may be an important contributor to the aging process. Genetically altered mice lacking DNA polymerase gamma (Polg) activity exhibited an elevated accumulation of random mtDNA point mutations, in conjunction with a severe deficiency in ATP synthesis and the early onset of aging-related phenotypes. However, these occurred in the absence of increased ROS production, protein carbonylation or mtDNA damage (66). Although there was no evidence for increased oxidative stress in this study, apoptotic signalling was significantly elevated in the Polg mice, and it is conceivable that areas of the cell with accumulated oxidative modifications may have been cleared away by cell death and subsequent autophagy processes. In future experiments, it would be interesting to determine whether the enhanced expression of Polg activity could result in extended lifespan in normal animals. A definite role for ROS in producing mutations and mitochondrial dysfunction was illustrated in a mouse model with compromised MnSOD activity and content. Age-related alterations observed included 25% decreases in complex I and V activities, a 50% increase in basal ROS generation and a 45% increase in 8-oxoG content (67). However, both the mean and maximum lifespan were not altered. As a result of this, there is considerable debate regarding the validity of the mitochondrial theory of aging. As Conley et al. reviewed, mitochondrial dysfunction can be observed in skeletal muscle before the detection of mtDNA mutations (68). In addition, the theory postulates that mitochondrial dysfunction is irreversible; however, much evidence exists to contradict this point. Clearly, more research is required, with a focus on when and how mtDNA mutations are involved with aging. Despite this, the associations between dysfunctional mitochondria, mtDNA mutations, and apoptosis remain strong themes in the description of mechanisms that may be causative to the aging process.

3.4.5. Uncoupling of oxidative phosphorylation

Coupling of the energy generated from electron transfer through the respiratory complexes to the synthesis of ATP is a major function of the mitochondrial network. However, the flow of

protons through complex V can be bypassed and redirected through protein channels which serve to uncouple respiration. The result of uncoupling is a decrease in ATP synthesis, despite increased oxygen consumption and respiratory rates (69). There is evidence which suggests that coupling is reduced with age. When compared with young individuals, coupling was lower by 50% in 30-month-old mice, resulting in decreased ATP production per O2 consumed (46). Another study supplemented this finding with the observation that uncoupling occurs in human skeletal muscle of subjects greater than 65 year of age that was accompanied with reduced ATP content (70). In the same study, it was determined that uncoupling affects muscles with a high type II fibre composition, compared with those that are composed of predominately type I fibers (41). Ghabi et al. also observed 21 and 40% decreases in the coupling of the IMF and SS mitochondria, respectively, in 36-month-old animals when compared with their younger counterparts (41). Potential causes for uncoupling of oxidative phosphorylation occurring with age may involve the increased activity and expression of uncoupling protein 3 (UCP3) that can be stimulated by oxidative stress. An increased activity of UCP3 has been proposed to lend protection to the cell, in response to increased oxidative stress that occurs with age. Indeed, mitochondria from UCP3 null mice demonstrated elevated levels of ROS production and oxidative modifications to cellular components. Whether the expression of uncoupling proteins in skeletal muscle is altered with aging is not well established. Some studies have observed a trend for increased UCP3 content (43), whereas others have suggested there is an age-related decrease or no change in this protein content (46, 71). Thus, if UPC3 content is not increased with age, it is likely that a greater proton leak with age could occur through increased permeability of the inner membrane by ROS-induced oxidative modifications of the lipid bilayer.

3.5. Potential of exercise to attenuate age-related mitochondrial dysfunction

Although it has long been established that exercise training increases, and muscle disuse decreases, the activity of mitochondrial oxidative enzymes in skeletal muscle, a lack of consideration of this notion in aging studies has led to discrepancies in our overall understanding of the effect of aging on muscle mitochondrial function. Indeed, some of the age-associated alterations found in mitochondrial activity can be the result of a reduction in the level of voluntary physical activity as individuals age (31). In this regard, it is notable that the adaptation to exercise is not limited to young individuals, because older athletes can increase the activity of mitochondrial oxidative enzymes as a result of training (72). This likely happens through increases in expression of the coactivator PGC-1a and the specific transcription factors NRF-1 and Tfam, the main regulators of organelle biogenesis and protein expression. One can assume that if mitochondrial function deteriorates with age, organelle biogenesis induced by exercise may attenuate this age-related decline, and therefore may have a protective role. However, despite the fact that exercise-induced increases in enzyme activities and mitochondrial content have been reported in aging individuals, less is known about the effects of exercise on the expansion of mtDNA mutations, ROS balance, and apoptosis in aged skeletal muscle. For example, in patients suffering from mitochondrial diseases due to mtDNA mutations, the introduction of an exercise program to improve muscle oxidative capacity and

mitochondrial function has been approached with caution. In those patients, exercise induced mitochondrial biogenesis but also increased both wild-type and mutant mtDNA, worsening the heteroplasmy ratio in muscle fibers (73). Thus, one might expect that this phenomenon could also occur in older individuals. However, in view of the evidence that chronic exercise can attenuate proapoptotic protein release from mitochondria in young animals, and reduce ROS production in intermyofibrillar mitochondria, it is worth investigating whether exercise can attenuate he enhanced apoptotic susceptibility evident in muscle from aged individuals.

Several lines of evidence support the fact that exercise may be beneficial in attenuating an aging-induced ROS imbalance. Old animals that were submitted to an 8-week treadmill exercise program, or 1 year of swimming, were found to have reduced oxidative damage compared with untrained old rats, notably due to alterations in antioxidant defences (74). At the mitochondrial level, recent work has revealed a 10% decrease in mitochondrial hydrogen peroxide production in animals as a result of lifelong voluntary wheel running (75). This may occur through the exercise-induced increase in mitochondrial content, a better redistribution of electrons through the electron transport chain, and (or) a better coupling between oxygen consumption and ATP synthesis in the exercised muscle of old animals. The precise mechanism for this effect remains to be determined.

4. Conclussion

Skeletal muscle is a remarkably adaptive tissue that is capable of changing its morphological, physiological, and biochemical properties in response to various perturbations. One of the most profound changes in skeletal muscle is mitochondrial biogenesis. Mitochondrial biogenesis is a very complex cellular process that requires the coordination of several mechanisms involving nuclear-mitochondrial corporation, mitochondrial protein expression and import, mtDNA gene expression, transcription factors activity, assembly of multisubunit enzyme complexes, regulation of mitochondrial fission and fusion as well as mitochondrial turnover. In turn, it seems with recognition of variant component of mitochondria of skeletal muscle; we can understand precisely the function of theses component in mitochondrial biogenesis process and effects of many interventions (e.g. Aging and diseases) on them. Also, we can comprehend the uncountable positive effects of exercise on these components. But, many vast and precise researches are needed to fully clarify these important issues.

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Clinical Relations

Clinical Aspects of Skeletal Muscle Modulators in Type 2 Diabetes Mellitus

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Additional information is available at the end of the chapter

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1. Introduction

Isotonic and isometric striated muscles contraction, as well as their extensibility and elasticity, are modulated by the central nervous system. Modulation at the level of neuromuscular synapses is also very compound, but precisely regulated, and essential for the induction of signals to muscle cells (Farahat & Herr 2010). The induced signal starts a cascade of multiple processes, necessary for sustaining muscle homeostasis. Without it, and also without muscle fuel utilization, muscle movement and skeletal support are deeply impaired. We therefore begin our description with electrochemical transmission, because its effects are subjected to modulation.

2. Calcium and ATP - mediators/modulators of ion channels in myocyte and its contractile activity

When a threshold potential is reached, an impulse travels along the muscle cell membrane and opens calcium gates in the membrane of the sarcoplasmic reticulum (SR). This is a trigger for the myofibrils and myofilaments located in the sarcoplasm, which turns the muscle "on". A muscle stops contracting, if the impulse is no longer being transferred from the neuron to the sarcolemma. At that moment, calcium gates close and calcium ions move back into the SR. At the terminal cisternae of SR with intracellular Ca^{2+} stores, the ryanodine receptor channel isoform 1 (RyR1) is located. It is an integral component of excitation–contraction coupling and the receptor activation plays a key role in this process. Excitation-contraction coupling may be triggered by a single-pulse of electrical stimulation and results in massive release of Ca^{2+} from the SR *via* the RyR1 to the cytosol after depolarization of the muscle plasma membrane. However, skeletal muscle function *in vivo* is generally regulated during repetitive firing of short trains of action potentials (Diaz-Sylvester et al., 2008).

The free cytosolic Ca^{2+} concentration in a skeletal muscle at rest is about 120 nM and in the millimolar range in its intracellular stores (Eltit et al., 2011). The RyR1 is activated by an increase in cytosolic Ca^{2+} levels (with peak activation at ~100 μM); regardless of the presence or absence of Mg^{2+}/ATP, and it is called calcium induced calcium release mechanism. A millimolar Ca^{2+} concentration also inactivates *per se* the RyR1. The receptor may also be inhibited by Mg^{2+} or activated by caffeine and ATP. The RyR1 channel is furthermore modulated by ryanodine, calmodulin, 12-kDa FK-506-binding protein (FKBP12) and so-called Homer proteins, as well as by various redox processes (Diaz-Sylvester et al., 2008; Eltit et al. 2011; Feng et al., 2008; Ye et al., 2012; Lawler et al., 2010).

Variations in the intracellular levels of endogenous RyR1 modulators are relevant to individuals with genetic disorders of skeletal muscles associated with mutations in the RyR1 of SR. Some of the disorders were investigated in malignant hyperthermia (MH) provoked by halothane. The RyR1 becomes more susceptible to activation by halothane, when Mg^{2+} levels decrease and/or Ca^{2+} levels increase in the cytosol. ATP binding greatly increases the RyR1 activity as well. Moreover, halothane-induced RyR1 activation is enhanced by increases in SR Ca^{2+} loads. Knowledge about cytosolic modulators may help us understand, why decreased Mg^{2+} sensitivity may result in higher sensitivity to halothane and why RyR1 carrying MH mutations are less sensitive to Mg^{2+} inhibition. Taking into account the fact that insulin decreases intracellular Mg^{2+} in muscles, we are able to explain why MH susceptibility has been associated with increased insulin levels (Diaz-Sylvester et al., 2008).

Another calcium channel is the dihydropyridine receptor (DHPR), serving as a voltage sensor in excitation-contraction coupling and a modulator of RyR1 through physical interactions (a conformational change of DHPR allosterically activates RyR1). In continuously depolarized skeletal muscle fibers, DHPR voltage sensors are inactivated and unable to initiate SR Ca^{2+} release. Both DHPR and RyR1 receptors bind with Triadin (a modulator from a family of proteins of the junctional sarcoplasmic reticulum) that is involved in many crucial aspects of muscle functions and E-C coupling in skeletal muscles (Diaz-Sylvester et al. 2008; Perez, 2011). Dihydropyridine-receptor agonists and antagonists can modulate both a single pulse electrical stimulation (which mainly triggers a fast calcium signal sensitive to RyR1) and tetanic stimulation (which induces a long-lasting, inositol triphosphate (IP_3)-generated signal) (Lawler et al., 2010).

Ca^{2+} release plays an essential role in initiating muscle contraction, increases glucose uptake (although its signalling is unresolved), activates numerous genes required for mitochondrial biogenesis, and results in ATP production in mitochondrial and cytoplasmic compartments during exercise (Hawley et al. 2006). A lasting contraction causes energy expenditure and ATP supply diminishes at some point (but usually no more than about 20 % of it even during very intensive exercise). This decline in ATP level causes muscle fatigue. The myosin heads remain bound to actin and can no longer swivel. Even when a nervous impulse is being transmitted to the muscle and calcium ions are still present, contraction/or strong contraction is not possible. Several different energy sources (phosphocreatine, muscle glycogen, blood glucose and fatty acids from adipose tissue) are used by myocytes to

maintain ATP levels in a working muscle. Although glucose is a predominant fuel for the muscles, diminished ATP levels in the myocyte are a signal to switch to the use of fat fuel, leading to an increased flux of free fatty acids and changes in the AMP/ATP ratio, contributing to fiber shifts. Fuel economy depends on the type of muscle fibres involved, the length of the physical effort and its type. There are high speed fibres which require carbohydrate fuels and generally used slowly contracting economical fibers with a fat-based aerobic metabolism (de Lange et al., 2007).

The AMP/ATP ratio is important for the activation of AMP–activated protein kinase (AMPK), which stimulates energy generating processes (glucose uptake and fatty acid oxidation). AMPK plays a key role in controlling muscle economy, inmaintaining muscle homeostasis, and in inducing a cascade of events within cells in response to the ever-changing energy charge of the cell (de Lange et al., 2007; Lira et al. 2010).

3. Modulators of the AMP–activated protein kinase relevant in type 2 diabetes mellitus

AMPK was discovered in skeletal muscles in 1995 (Verhoeven et al., 1995). It is a heterotrimeric enzyme comprised of two catalytic (α1 or α2) subunits and two regulatory (β1 or β2 and gamma1, gamma2, or gamma3) subunits (all are encoded by separate genes). AMPK activity inhibited preparations of acetyl-CoA carboxylase (ACC) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) was first discovered. Later, it was noticed that AMPK activity can also be regulated by physiological stimuli, independent of the energy charge of the cell (like hormones and nutrients) (Lim et al., 2010; Steinberg & Kemp, 2009).

AMPK is activated during contraction. Its modulation (related to the muscle energy state) depends on two distinct mechanisms – on an AMP-dependent pathway mediated by kinase LKB1 and on a Ca^{2+}-dependent pathway mediated by the calmodulin-dependent protein kinase (CaMKKb). AMPK in skeletal muscles increases fatty acid oxidation and mitochondrial biogenesis (Lim et al., 2010). These processes require phosphorylation of the peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) and SIRT1. The last protein works as an NAD^+-dependent protein deacetylase or ADP-ribosyltransferase. It also activates PGC-1α involved in fatty acid utilization and mitochondrial respiration (de Lange et al., 2007; Zhang et al., 2009).

Mitochondrial dysfunction observed in type 2 diabetes mellitus leads to a decrease in ATP levels and possibly to changes in NAD^+/NADH levels. It can influence methylenetetrahydrofolate dehydrogenase ($NADP^+$) activity, which depends on the concentration of NAD^+ and on the ATP/ADP ratio (Zhang et al., 2009; Mailloux & Harper, 2010). NADH inhibits and βNAD activates AMPK, but NAD is a much weaker activator than AMP (we also do not know, if AMP is a direct activator). AMPK activity is modulated not only by nucleotides, but also by kinases, fatty acids, insulin, cytokines and reactive oxygen species (ROS) (Rafaeloff-Phail et al., 2004; Steinberg & Kemp, 2009; Zhang et al., 2009).

The major glucose transporter - GLUT4 translocation to sarcolemma, is also a result of AMPK-activated PGC-1α, where SIRT1 activity is required (Steinberg & Kemp, 2009). This pathway of glucose uptake in skeletal muscles is stimulated by palmitate (PA) (regardless of AMP concentrations) (Pu et al., 2011). It takes place only when skeletal muscles are exposed to PA for a short time. Chronic exposure to PA blocks Akt phosphorylation and inhibits insulin induced glucose uptake. Besides PA, linoleic, oleic, and stearic acids can stimulate AMPK and Akt (Pu et al., 2011).

5-Aminoimidazole-4-carboxyamide ribonucleoside (AICAR) is another AMPK activator increasing GLUT4 translocation to sarcolemma and glucose uptake in rats' skeletal muscles. AICAR as well as physical effort, leads to AMPK activation, which enhances a specific protein fatty acid translocase – FAT/CD36 in sarcolemma. Either AICAR or physical effort, stimulate AMPK activity in obese and type 2 diabetes mellitus, which can further enhance muscle lipid breakdown and the capacity for ATP generation (by the activation of mitochondrial fatty acid β-oxidation enzymes) (de Lange et al., 2007; Steinberg & Kemp, 2009).

Physical effort enhances the production of ROS. ROS is produced continuously in mitochondria and its level is reduced by the activation of AMPK. This process has especially been investigated in smooth muscles, because oxidative stress (e.g. induced by free fatty acids) in patients with a metabolic syndrome contributes to the development of cardiovascular diseases (Steinberg & Kemp, 2009).

In response to the contraction of a myocyte, interleukin-6 (IL-6) is produced and released. Although AMPK participates in the regulation of IL-6 release from oxidative muscle, IL-6 significantly increases AMPK activity in myotubes as well as enhances fatty acid oxidation and insulin-stimulated glucose uptake.

The effect of adiponectin on AMPK activation is not well known. AMPK is probably indirectly activated by increases in levels of intracellular ATP, but it is known that leptin directly stimulates the α2 and β2 catalytic subunits expression of AMPK in skeletal muscle. This occurrence correlates with enhanced fatty acid oxidation and the inhibition of lipogenesis. Such leptin action was reported in healthly individuals, but AMPK in obese individuals was not activated by leptin.

Cytokines may also inhibit AMPK activity - tumor necrosis factor α (TNFα), which signals through the TNF receptor (TNFR) 1, upregulates protein phosphatase 2C (PP2C) and suppresses AMPK. This in turn suppresses fatty-acid oxidation and increases intramuscular diacylglycerol accumulation in skeletal muscle.

AMPK is a major cellular energy sensor and a master regulator of metabolic homeostasis - several processes necessary for the mechanical function of muscles are controlled by 1 enzyme. Today, AMPK is already the target of several classes of drugs used for type 2 diabetes mellitus, including metformin and tiazolidinediones (Steinberg & Kemp, 2009; Zhang et al., 2009).

4. Myocyte modulators and insulin resistance

Adipose tissue protects other cell tissues from "lipotoxicity", but obesity causes dysregulation of lipolysis and lipid oversupply into muscles and other tissues. Adipose tissue also informs the brain and other tissues about this dysregulation through adiponectin, leptin, resistin, IL-6 and TNFα (Steinberg & Kemp, 2009). Not only is the adipose tissue recognized as an endocrine organ, it also produces modulators relevant for IR. There is also the hepatic insulin sensitizing substance (HISS), which after being released from the liver, acts on skeletal muscles to stimulate glucose storage in the form of glycogen. The intramyocytal signaling cascade in type 2 diabetes mellitus is impaired due to insulin modulation by HISS and in the future we will probably differentiate these two types of diabetes mellitus in the GP's surgery (Lautt et al., 2010).

It is clear that insulin activates the pathway of kinase Akt and hampers AMPK activity, but despite the developing knowledge about AMPK, we still do not know enough about IR. When skeletal muscles become unable to switch between glucose and fatty acid use, the cause of glucose uptake and utilization is dramatically lessened.

There is evidence that nitric oxide (NO) is a modulator of insulin action and insulin resistance is linked to inducible NO synthase (iNOS) induction in skeletal muscle cells (and in other insulin target cells as well). An increased expression of multiple inflammatory cytokines (TNFα, interferon-c, IL-6, and IL-1β) observed in obesity has to induce iNOS as it was shown on an animal model of acute systemic inflammation that iNOS in muscle (also in liver and adipose) tissue was induced by the administration of the endotoxin lipopolysaccharide (LPS). Cytokines modulate glucose transport in the skeletal muscle by inducing the expression of iNOS. Moreover, a high-dose of aspirin improves glucose metabolism in patients with type 2 diabetes mellitus. There is also genetic evidence that iNOS mediates skeletal muscle IR in obese high-fat fed mice and that the induction of iNOS in skeletal muscle modulates whole-body glucose metabolism (Cha et al., 2011; Soskić et al., 2011).

AMPK phosphorylates and activates NOS, and physiological levels of NO can stimulate glucose uptake and oxidation in human skeletal muscles, but the meaning of NOS for glucose homeostasis is still cloudy (Steinberg & Kemp, 2009). Physical effort increases NOS activity and the expression of neuronal (nNOS) and endothelial (eNOS) NOS. There is some evidence that mitochondrial respiration in myocytes is inhibited by NOS activity. NO donors, such as sodium nitroprusside, raise skeletal muscle cGMP content and increase glucose uptake in rats, but the AICAR effect is not blocked by NOS inhibitors (McConell & Wadley, 2008). Chronic administration of NOS inhibitors (monomethylarginine - L-NAME) in drinking water decreases glucose tolerance in rats (Balon et al., 1999).

Another therapeutic target in insulin resistance may be stearoyl-CoA desaturase (SCD). The major product of SCD - oleic acid, may play an important role in the regulation of intracellular ceramide synthesis, which is recognized as an insulin-desensitizing lipid molecule. The lack of the SCD1 gene increases the rate of fatty acid ß-oxidation - it results in a decrease of free fatty acids and long-chain fatty acyl-CoAs content in the soleus and red gastrocnemius SCD1-/- mice models. The AMPK pathway is also involved in this process. It

increases this enzyme's phosphorylation and contributes to an increase of insulin sensitivity in the muscles. The expressions of a protein-tyrosine phosphatase 1B (PTP-1B) is down regulated in the case of SCD1 deficiency. This expression is responsible for the sustained insulin receptor autophosphorylation (Dobrzyn & Dobrzyn, 2006; Dobrzyn et al., 2010).

PUFA, cholesterol, vitamin A, hormonal signals (e.g. insulin, glucagon), environmental and developmental factors (temperature changes, metals), alcohol, thiazolinediones, peroxisomal proliferators modulate stearoyl-CoA desaturase (SCD) expression in adipose tissue and liver. It is proven that some of these compounds are able to modulate SCD by catalyzing the synthesis of monounsaturated fatty acids (mainly oleate and palmitoleate acids) in muscles. Glucose is probably the most important modulator of SCD expression in the skeletal muscle. Its long-term oversupply increases SCD expression and enzyme activity - it leads to fast and specific changes in fatty acid metabolism. Further investigations of SCD modulators may be relevant, because stress and inflammatory stimuli can also modulate Transcriptional regulation of SPT, which is the first committed step in *de novo* ceramide synthesis (Dobrzyn & Dobrzyn, 2006).

There is also evidence that insulin resistance and type 2 diabetes mellitus are linked to the reduction of insulin receptor substrate (IRS-1) expression, and the impairment of PI3K and kinase Akt activity in skeletal muscle. Impaired insulin-mediated glucose transport is a characteristic defect in type 2 diabetes mellitus. There is normal expression of the human GLUT4 gene in type 2 diabetes mellitus, but a cause of insulin resistance is searched out in traffic of this glucose transporter to sarcolemma. The translocation of GLUT4 to the membrane increases its glucose permeability. GLUT4 intracellular redistribution is directly linked to a signaling protein known as AS160 which may be activated by both the insulin pathway and AMPK. Hampering of the Akt substrate of 160 kDa (AS160), phosphorylation (as a result of Akt phosphorylation in the position of Thr308) diminishes the activity of AS160. Impaired insulin-stimulated phosphorylation of AS160 has been discovered in patients with insulin resistance or/and type 2 diabetes mellitus (Treebak et al., 2007).

When no muscles stimulation exists, then only about 10% of GLUT-4 transporters are located in the sarcolemma, but their amount increases to 50% after 2-3 minutes of stimulation with insulin. Relocation of GLUT4 from an intracellular pool of myocytes to its membrane is phosphorylated and activated not only by insulin (associated with the activation of kinase PI3K), but also may be activated by IGF1, EGF and AICAR. NO induces the upregulation of GLUT4 mRNA (by the AMPK dependent pathway) (Wang et al., 1996; Russell et al., 1999).

The role of NO in a skeletal muscle has still not been investigated enough. The interaction of AMPK, NOS enzymes, and NO levels in skeletal muscles is intriguing (AMPK activates eNOS and nNOS, but AMPK activation inhibits iNOS expression). We think that the significance of the effect of SCD1 deficiency/downregulation on lipid metabolism and insulin sensitivity in the skeletal muscle is underrated, as lipids are transported through sarcolemma (Steinberg & Kemp, 2009).

The last investigation showed that dysregulation in PA uptake and reduced CD36 protein level in murine myocyte are recognized as a reason of metabolic complications analogous to

"the cardio-metabolic syndrome" in patients treated with a protease inhibitor of the human immunodeficiency virus (Richmond et al., 2010).

It is a real challenge to establish a cause of diabetes mellitus type 2, which is probably multifactorial. One of those factors is a sedentary lifestyle, another one is obesity and a high fat calorie diet, they induce inflammatory cytokines and reduce insulin signaling. This subsequent loss of homeostatic signaling yields a low heat shock proteins (HSP) state (Atalay et al., 2009). The HSPs respond to a wide variety of stress factors (e.g. elevated temperature, hypoxia, altered pH) and first studies in patients with diabetes mellitus type 2 revealed that hot-tub therapy is able to improve glycemic control (Gupte et al., 2009, as cited in Hooper, 1999). Later it was described that HSP90 maintains the Akt and plays an important role in the regulation of glucose transport mediated by insulin (Atalay et al. 2009, as cited in Sato S. et al. 2000). Moreover, a lower level of HSP72 was found in skeletal muscles of patients with type 2 diabetes and some HSPs were strongly induced in skeletal muscle with physical exercises especially in fast-twitch muscles (Atalay et al. 2009, as cited in Bruce, 2003 and Kurucz, 2002). A recent study demonstrated that heat treatment protects skeletal muscles in rats from a high-fat diet–induced insulin resistance and provided strong evidence that HSP induction in skeletal muscles could be a potential therapeutic treatment for obesity-induced insulin resistance (Gupte et al., 2009).

The HSP60 expression improves mitochondrial dysfunction observed in skeletal muscles of patients with type 2 diabetes as measured by oxidative capacity, with a high-fat diet in parallel with increased mitochondrial protein expression (Gupte et al., 2009). Also thiazolidinediones increase expression of an array of mitochondrial proteins in diabetic mousses (it also concerns PGC1α, the master regulation of mitochondrial biogenesis) (Cantó et al., 2009) and we think that mitochondria are a primary target of heat therapy from oxidative stress.

Recently, BGP15 (an HSP inducer) was found as a potent insulin sensitizer, which increases muscle glucose utilization (Literáti-Nagy et al. 2009). It protects the mitochondrial membrane against oxidative damage by the activation of PI-3-kinase – Akt pathway. This well-known cytoprotective pathway is suppressed by Poly (ADP-ribose) polymerase (PARP), which can induce rapid cellular NAD^+ and ATP pool depletion leading to mitochondrial dysfunction. The small molecule BGP15 is an inhibitor of PARP presented in eukaryotes and may be a novel strategy for therapies of insulin resistance (Halmosi et al. 2001)

We were able to find more evidence that our surroundings also have input in type 2 diabetes mellitus etiology. Bisphenol-A (BPA), a component of polycarbonate plastics and epoxy resins, widely used in multiple consumer products like plastic food/fluids containers is commonly recognized as a disruptor of glucose homeostasis, which provokes hyperinsulinemia and insulin resistance. The measurable levels of this synthetic estrogen were reported in human serum, urine, breast milk, fetal plasma, and placental tissue. It probably modulates the glucose transporter GLUT4 through two estrogen receptors (ERα and ERβ). Studies showed that elevated BPA level was associated with elevated hemoglobin A1c (HbA1c). Also higher urinary BPA level was found to be associated with diabetes type 2 diabetes mellitus independently of traditional diabetes risk factors (Lang et al., 2008; Vandenberg et al., 2007; Li et al., 2012). On the other hand, α-lipoic acid (LA) (found abundantly in green vegetables)

affects the insulin-signaling cascade and protects myocyte from oxidative stress-induced insulin resistance (Maddux et al., 2001). Also green and black teas suppress insulin resistance by retaining the expression of GLUT4 in skeletal muscle. Epidemiological data suggests that drinking green tea is negatively associated with type 2 diabetes (Anderson & Polansky 2002; Nishiumi et al 2010; Yan et al. 2012). Green tea reduces oxidative stress in animal adipocytes (Yan et al. 2012), it also decrease ROS content of cultured mouse myotubes (Buetler et al., 2002), but we have not yet investigated, if green tea protects myocytes from oxidative stress-induced insulin resistance. Several international prospective studies show a protective effect of coffee on the development of type 2 diabetes (Kempf & Martin 2010). We focused on the modulators crucial for peripheral insulin resistance, but impaired pancreatic glucose sensing is involved in etiology of diabetes mellitus type 2 as well. It is a genetically complex chronic disease, but lifestyle changes can delay progression to diabetes, even in high-risk individuals, for at least a decade (Cho et al. 2012; Knowler WC et al. 2009).

5. Muscular contraction – underestimated modulator

Physical exercise is a potent stimulus for increasing glucose uptake in the complete absence of insulin. This mechanism may be modulated by AMPK, Akt/protein kinase B phosphorylation, NO and Ca^{2+}/calmodulin-dependent protein kinase (CaMK) and protein kinase C (PKC) and is initiated by acute exercise. This is a short–lived effect, which disappears in 16-48 h. However, repeated physical exercise results in an increase in insulin action, sustain in skeletal muscle of patients with insulin resistance and type 2 diabetes mellitus. This improvement in insulin sensitivity is related to increasing AS160 phosphorylation, expression and/or activity protein kinase B (Akt) and AMPK (Hawley & Lessard, 2008; Ismail-Beigi, 2012; Cahová et al., 2007; Wasserman et al., 2011).

Exercise training results also in glucose uptake through an increase in the level of GLUT4 trafficking. Training may cause increasing 30% of GLUT4 in sarcolemma of patients with type 2 diabetes mellitus and 20% of GLUT4 in control group (Wasserman et al., 2011). We outlined above, how muscle contraction my improve insulin sensitivity by increased lipid oxidation. There is also clinical evidence that physical activity (150 min/week) and diet-induced weight loss of 5–7% are able to reduce the risk of progression from impaired glucose tolerance to type 2 diabetes mellitus. Therefore, very recently the guidance in type 2 diabetes mellitus has been changed – more attention is given to muscle contraction as a modulator, which has become a key part of type 2 diabetes mellitus self-management (Sigal et al., 2006).

6. Conclusion

Type 2 diabetes mellitus is still a challenge for biochemists, clinicians and epidemiologists. It has been assessed that 23.6 million people lived in the US in 2007 with diagnosed and undiagnosed diabetes (7.8% of the general population). There are forecasts that 366 million people in the world will live with type 2 diabetes mellitus in 2030 (Zhang et al., 2009).

Recently, physical exercise has been recognized by health organizations as an undoubted modulator of glucose uptake in skeletal muscles. We hope that awareness of this fact will be

created by promotion in the GP's surgery and through mass media. Muscle contraction as a modulator of GLUT4 expression, which also improves insulin signal transduction at the level of PI3K and AS160, and activates AMPK, is able not only to diminish a dose of oral medicine in type 2 diabetes mellitus, but also to influencethe future of patients with insulin resistance.

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Chapter 12

Skeletal Muscle Mitochondrial Function/Dysfunction and Type 2 Diabetes

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Additional information is available at the end of the chapter

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1. Introduction

"Let food be your medicine and medicine be your food" stated Hippocrates, the father of Western medicine, in 400 B.C. This statement was based on the belief that food was able to influence disease, a concept that was revived several times in later years by painters, writers, scientists, and philosophers. One such philosopher, Ludwig Feuerbach, famously wrote in his 1863-4 essay "man is what he eats" introducing the idea that if we want to improve the spiritual conditions of people we must first improve their material conditions (Feuerbach, 2003). However, for years his warnings remained unheeded, at least in Western countries, in contrast to the teachings of Indian and Chinese medicine which for millennia have argued that a living organism has to assume a healthy diet. Like diet, physical activity has been also considered an important starting point for people's health. Hippocrates wrote in his book *Regimen* "if we could give every individual the right amount of nourishment and exercise, not too little and not too much, we would have found the safest way to health" (Hippocrates, 1955). Our knowledge about the links between diet, exercise, and disease has vastly increased since Hippocrates time. A healthy lifestyle based on diet and physical activity is now considered the keystone of disease prevention and the basis for a healthy aging. However, modern society has created conditions with virtually unrestricted access to food resources and reduced physical activity, resulting in a positive overall energy balance. This is far from the environment of our "hunter-gathered ancestros" whose genes were modulated over thousands of years adapting our metabolism to survive when food was scarce and maximizing energy storage when food became available. In terms of evolution, this radical and sudden lifestyle change in modern society has led to a dramatic increase in the incidence of metabolic diseases including obesity and type 2 diabetes mellitus (T2DM). It seems clear that the development of T2DM has a genetic component that becomes obvious when individuals are exposed to western lifestyle. However, environment

plays a critical role in the incidence of the disease being obesity the main etiological cause of T2DM. Thus, modest weight loss is enough for obese glucose intolerant subjects to prevent the development of T2DM (National Task Force on the Prevention and Treatment of Obesity, 2000).

T2DM also known as "non-insulin-dependent diabetes mellitus" or "adult-onset diabetes", is a metabolic disorder characterized by high blood glucose, insulin resistance, and relative insulin deficiency. T2DM is diagnosed when fasting blood glucose levels are higher than 126 mg/dL (7.0 mM) or the two-hour blood glucose levels higher than 200 mg/dL (11.1 mM) after a glucose tolerance test. T2DM is now considered to be a global epidemic with significant social and economic consequences both at the individual and population level. The International Diabetes Federation estimates that 366 million people suffered from this disease in 2011 and predicts that these numbers will increase to 552 million people by 2030. Risk factors for T2DM include genetic predisposition as well as environmental factors, including adverse intrauterine environment, inactivity, diet, obesity, and aging (International Diabetes Federation, http://www.idf.org/diabetesatlas/news/fifth-edition-release).

The term "prediabetes" is used to describe a condition characterized by impaired glucose tolerance or impaired fasting glucose (Pour and Dagogo-Jack, 2011). The pathophysiology of prediabetes is characterized by alterations in insulin sensitivity and pancreatic beta-cell function, usually associated with increased adiposity (Dagogo-Jack et al., 2009). According to the World Health Organization (WHO) and the American Diabetes Association (ADA), impaired glucose tolerance is defined as a two-hour plasma glucose level between 140 and 199 mg/dL (7.8 to 11.0 mmol/L) after an oral glucose tolerance test. In this condition, fasting glucose levels may be either normal or mildly elevated. The ADA defines a state of impaired fasting glucose when fasting plasma glucose levels are over 100 mg/dL (5.6 mmol/L) but less than 125 mg/dL (6.9 mmol/L). Importantly, subjects with prediabetes are at higher risk for progressing to diabetes. Fortunately, such progression is not inevitable and can be delayed or prevented through pharmacological and lifestyle interventions based on diet and exercise (Knowler et al., 2002, Knowler et al., 2009, Tuomilehto et al., 2001). While these data are encouraging, these interventions are costly, require a very high degree of commitment of the subjects, and are not always successful. Although the progress in understanding the metabolic derangements of T2DM has led to significant advances in the treatment of this disease, it remains unclear whether current therapeutic approaches can really improve the underlying metabolic defects. Therefore, there is an urgent need to characterize the complex pathophysiology of the disease, to identify and target specific mechanisms in order to slow down the worldwide diabetes epidemic.

2. Insulin action and insulin resistance

Insulin essentially provides an integrated set of signals that allow for the balancing of nutrient availability and caloric demands (Samuel et al., 2010). In collaboration with the opposing hormone glucagon, it is responsible for maintaining glucose homeostasis, which is necessary to ensure proper function and survival of all organs. The regulation of plasma glucose concentrations is vital for the entire body and both hypoglycemia and hyperglycemia can impair whole-body physiology, ultimately leading to cellular death. This

is why it is critical to regulate and maintain plasma glucose levels around 5mM, the physiological set point in mammals (Saltiel, 2001).

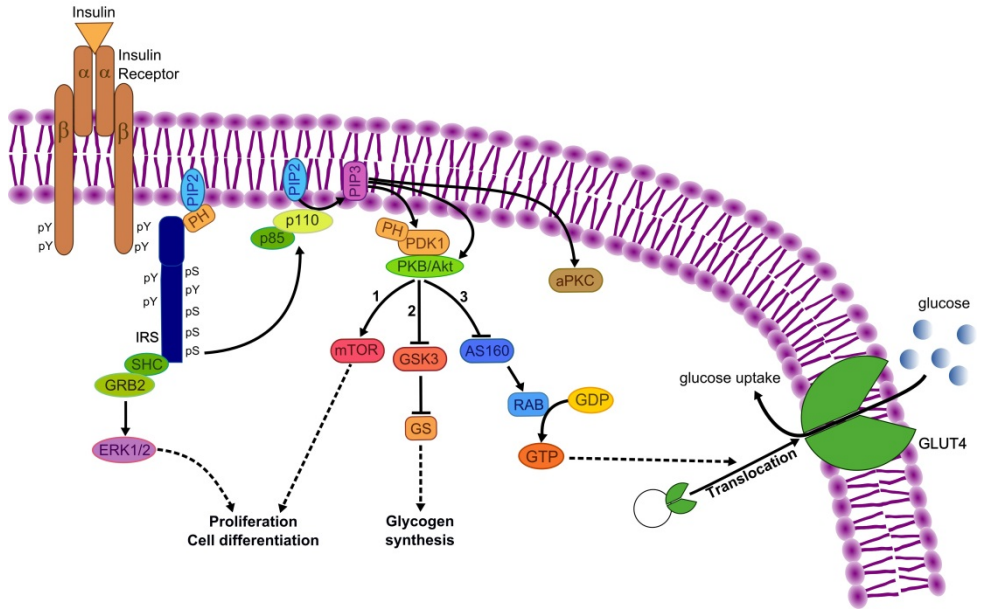
The primary targets of insulin action to maintain glucose homeostasis are skeletal muscle, liver, and adipose tissue. Under physiological conditions, carbohydrates provided by the diet increase plasma glucose levels and promote insulin secretion from pancreatic β cells of the islets of Langerhans. Once secreted, insulin binds to its receptor, triggering a cascade of downstream phosphorylation events that expand the initial signal (*Figure 1*). Insulin binds to its receptor and activates its intrinsic protein tyrosine kinase activity, resulting in the phosphorylation of tyrosine residues located in the cytoplasmic face. The activated receptor, in turn, recruits and phosphorylates a group of substrate molecules. They have the role of docking proteins and are known as "insulin receptor substrates" (IRS). Among these, IRS1 and IRS2 appear to be the major adapter molecules that play a role in insulin cascade. IRS1/2 can activate different intracellular processes, above all glucose metabolism and mitogenesis. Once phosphorylated, these docking proteins recruit the heterodimeric p85/p110-PI3K at the plasma membrane: the regulatory subunit p85 binds to IRS1/2 and this event allows the activation of the catalytic subunit p110, which produces the lipid second messenger PIP3 from PIP2. PIP3 activates a serine/threonine phosphorylation cascade of PH-domain containing proteins: PDK1, the serine/threonine protein kinase B (PKB)/Akt and the atypical protein kinases C ζ and λ isoforms (aPKC ζ - λ). Specifically, PKB phosphorylation causes:

1. activation of the mammalian target of rapamycin (mTOR), an intracellular protein as well as a critical component of the PI3K/AKT pathway, that acts as a central regulator of multiple signaling pathways that mediate growth, proliferation and cell differentiation;
2. glycogen synthase kinase-3 (GSK3) inactivation. This event relieves the inhibitory phosphorylation of glycogen synthase (GS), which becomes activated and promotes glycogen synthesis;
3. insulin-stimulated translocation of the glucose transporter GLUT4 at the plasma membrane, resulting in increased glucose uptake. This pathway involves the protein AS160/TBC1D4. AS160 normally inhibits translocation of GLUT4 through its interaction with RabGTPase protein. The inhibitory phosphorylation of AS160 favors the GTP-loaded state of Rab and relieves the inhibitory effect on GLUT4, stimulating its translocation to the plasma membrane. In this way, insulin can promote the docking and fusion of GLUT4-containing vesicles to the plasma membrane and finally stimulate glucose uptake.

Insulin action in normal conditions differs depending on the target tissue:

- in skeletal muscle, it increases glucose transport, increasing glucose uptake and activating glycogen synthesis;
- in liver, it promotes glycogen synthesis and *de novo* lipogenesis, while inhibiting gluconeogenesis;
- in adipose tissue, it suppresses lipolysis and promotes lipogenesis, leading to a net increase in lipid accumulation.

Insulin secretion from pancreatic β cells is suppressed during fasting. During this state, there is an increase in hepatic glucose production and glycogenolysis. Lipid synthesis diminishes in liver while lipolysis increases in adipose tissue.



IRS, insulin receptor substrate; S, serine; Y, tyrosine; PH, pleckstrin homology domain of the IRS-1; SHC, Src Homology 2 domain; GRB2, growth factor receptor-bound protein 2; ERK, extracellular-signal-regulated kinases or classical MAP kinases; PIP2, phosphatidylinositol 4,5-bisdiphosphate; PIP3, phosphatidylinositol 3,4,5-tridiphosphate; PDK1, phosphoinositide-dependent protein kinase 1; PKB/Akt, protein kinase B; mTOR, mammalian target of rapamycin; GSK3, glycogen synthase kinase 3; GS, glycogen synthase; AS160, 160 kDa Akt substrate; GDP, guanosine diphosphate; GTP, guanosine triphosphate; aPKC, atypical protein kinase C.

Figure 1. Insulin signaling pathway

3. Pathogenesis of Type 2 Diabetes Mellitus

An important early phenotype associated with increased T2DM risk is insulin resistance. Insulin resistance, defined as reduced responsiveness to the effects of insulin to promote glucose disposal into muscle, liver and adipose tissue, is present in high-risk individuals years before the onset of T2DM, and can predict the development of the disease (Martin et al., 1992, Tabak et al., 2009). Given these data, it is alarming that the high prevalence of insulin resistance in the population predicts further dramatic increases in the worldwide epidemic of T2DM. Individuals with established T2DM show several physiological abnormalities, including elevation in fasting glucose levels, elevation in postprandial glucose levels, or both. Insulin resistance in adipose tissue, skeletal muscle, and liver, together with pancreatic beta-cell dysfunction represent the core pathophysiologic defects of T2DM (DeFronzo, 1988).

In the initial stages of development of T2DM, insulin is not able to correctly stimulate skeletal muscle glucose uptake after carbohydrate intake, leading to postprandial hyperglycemia. In adipose tissue, the major fat storage tissue in mammals, insulin resistance results in increased lipolysis and fatty acid release. Increased circulating fatty acids decrease

the ability of insulin to suppress hepatic glucose production and allow a constant increase in fatty acid synthesis. This dysregulation of carbohydrate and lipid metabolism accelerates the progression of insulin resistance. During the first stages of the development of the disease, pancreatic beta-cells have the ability to compensate for insulin resistance by increasing basal and postprandial insulin secretion to correct hyperglycemia. When pancreatic beta-cells can no longer compensate they become unable to respond appropriately to glucose levels. This pancreatic beta-cell failure leads to the deterioration of glucose homeostasis and the development of T2DM. This pattern of physiological abnormalities in skeletal muscle, adipose tissue, liver, and pancreas presents itself in the late stages of the disease (Saltiel, 2001). Additionally, abnormal secretion and regulation of incretins in the gastrointestinal tract, hyperglucagonemia due to alterations in pancreatic alpha-cells, increased glucose reabsorption in kidney, and altered balance of central nervous system pathways involved in food intake and energy expenditure play an important role in the development of T2DM (Defronzo, 2009). This complex pathophysiology makes difficult to identify the primary events responsible for the development of T2DM.

4. Skeletal muscle insulin resistance and T2DM

As mentioned above, insulin resistance is a key component for the development of T2DM. However, the underlying molecular mechanisms are still unclear. Himsworth and Kerr, using a combined oral glucose and intravenous tolerance test, were the first to demonstrate that tissue-specific insulin sensitivity was lower in T2DM individuals (Himsworth, 1940). Ginsberg and colleagues provided another important evidence related to the decreased ability of insulin to promote glucose uptake in subjects with T2DM (Ginsberg et al., 1975). Later on, clear evidences about skeletal muscle insulin resistance in T2DM subjects were provided by DeFronzo and colleagues, who used the euglycemic-hyperinsulinemic clamp technique to quantify insulin-stimulated glucose uptake. With this technique, in a series of studies DeFronzo and colleagues demonstrated that both lean and obese T2DM subjects have marked decrease in whole body glucose disposal during the insulin clamp (DeFronzo, 1988). Skeletal muscle is the largest insulin-sensitive organ in humans accounting for more than 80% of insulin stimulated glucose disposal (DeFronzo et al., 1985). Therefore, insulin resistance in this tissue has major consequences on whole-body metabolic homeostasis.

Several mechanisms have been proposed as potential contributors to insulin resistance in skeletal muscle, including accumulation of intracellular lipid derivatives (diacylglycerol and ceramides), endoplasmic reticulum stress, impaired gene transcription, and pro-inflammatory signals (Ozcan et al., 2004, Straczkowski et al., 2007, Patti et al., 2003, Timmers et al., 2008, Sell et al., 2006). Moreover, several evidences linked mitochondrial defects to insulin resistance and T2DM (Lowell and Shulman, 2005), suggesting that these organelles are key players in maintaining energy homeostasis.

In this chapter we will discuss the potential role that mitochondrial dysfunction plays in T2DM etiology. In addition, a critical review of the current status of the topic will be

presented. In order to facilitate the reader the understanding of this chapter content we will briefly introduce several aspects of skeletal muscle composition, metabolism, mitochondria biogenesis and regulatory machinery that are necessary to comprehend subsequent information.

4.1. Skeletal muscle fiber types and metabolism

Skeletal muscle is a complex tissue composed of different fiber types, which have distinct mechanical and metabolic properties. Adult mammalian skeletal muscle is organized in motor units. Each of these functional systems is composed of a motor neuron and a group of muscle fibers. There are four major fiber types in mammalian skeletal muscle, categorized based on their myosin heavy chain (MyHC) composition: type 1 (slow oxidative), type 2A (fast-twitch oxidative), type 2X (fast-twitch oxidative-glycolytic), and type 2B (fast-twitch glycolytic). In adult human skeletal muscle type 2B fibers are not detectable and the oxidative capacity of type 2X fibers is lower than that observed in rats and mice (Schiaffino and Reggiani, 2011). For additional reading we recommend a review published in Physiological Reviews written by Stefano Schiaffino and Carlo Reggiani that provides an up to date and detailed understanding of this topic (Schiaffino and Reggiani, 2011). For the purposes of this discussion, it is important to keep in mind skeletal muscle diversity: distinct skeletal muscle fibers differ in their energy requirements for cellular function, including contractile activity. Energy is provided by adenosine triphosphate (ATP) hydrolysis to adenosine diphosphate (ADP) and inorganic phosphate (Pi). ATP can be generated by three main mechanisms that vary in their capacity and velocity to resynthesize ATP. The Phosphocreatine (PCr)-creatine kinase (CK) system corresponds to a high-power and low-capacity ATP production reservoir. Glycolysis is the metabolic process by which glycogen and glucose are metabolized to pyruvate and subsequently to lactate; this process has a lower power but a higher capacity for ATP generation than the PCr-CK system. The other energy production resource is the mitochondrial oxidative phosphorylation system, which can obtain ATP from different substrates: pyruvate, fatty acids, amino acids, and ketone bodies. The oxidative phosphorylation system has a very high capacity for ATP generation but a lower power when compared to the other two ATP production systems. It is also important to highlight that mitochondrial mediated ATP resynthesis is highly dependent on oxygen and substrate availability.

Due to its intrinsic characteristics, slow and fast muscle fibers differ in their relative contribution to energy production from PCr-CK, glycolysis, and oxidative phosphorylation processes. The relative contribution of these metabolic pathways is mostly established during differentiation according to the specific function and energy demands of each fiber type. Moreover, it is important to mention that adult skeletal muscle fibers show what has been termed “metabolic flexibility.” Metabolic flexibility is the systemic capacity to switch between different substrates for ATP production depending of their availability and energy requirements (Kelley et al., 1999, Storlien et al., 2004). Thus, skeletal muscle is able to predominantly utilize both glucose and free fatty acids as fuel sources for energy production. The utilization of these two energy sources depends on the fasting/feeding state of the individual:

- During fasting state, muscle glucose uptake is low and plasma fatty acid concentration is elevated due to lipolysis in adipose tissue. Thus, under fasting conditions, fatty acids represent the main source for energy production in skeletal muscle.
- During feeding state, plasma glucose concentration increases and stimulates insulin secretion that exerts two principal and simultaneous actions: suppression of lipolysis in adipose tissue, leading to a reduction in plasma fatty acid concentration, and stimulation of glucose uptake in skeletal muscle. This event, together with the activation of key enzymes in glucose metabolism, leads to a marked increase in muscle glucose oxidation. After glucose is transported into the myocytes trough the GLUT4 transporter, it is immediately phosphorylated by hexokinase II, and the phosphorylated glucose is stored as glycogen or enters the glycolytic pathway for energy production. Thus, during feeding conditions, the main source for energy production in skeletal muscle is glucose.

Therefore, muscle energy metabolism has to be capable of switching from predominant oxidation of fatty acids during fasting state, to predominant oxidation of glucose during feeding state. However, obese and type 2 diabetic subjects are unable to shift between substrates (fatty acids or glucose) demonstrating a high degree of metabolic inflexibility (Kelley et al., 1999). This inability to oxidize one substrate or another results in impaired glucose and fatty acid storage as glycogen and triglycerides, respectively. These concepts of metabolic flexibility and inflexibility are documented by studies performed by Dave Kelley and co-workers (Kelley et al., 1999, Kelley et al., 2002a, Kelley et al., 2002b) and summarized by Storlien et al. in the "Proceedings of the Nutrition Society" (Storlien et al., 2004).

4.2. Pathogenesis of Insulin Resistance in Skeletal Muscle

Both obese subjects with or without T2DM have marked skeletal muscle insulin resistance compared to lean non-diabetic subjects. The severity of the insulin resistance positively correlates with BMI (DeFronzo, 1982, Wedick et al., 2009). The mechanism through which obesity causes insulin resistance in skeletal muscle seems to be associated with the accumulation of fatty acids in the myocytes. Among the various types of fatty acids, saturated long-chain ones, including palmitic and stearic acids, are strong inducers of insulin resistant state (Hirabara et al., 2009). Obese subjects with or without T2DM are characterized by an increase in plasma fatty acid concentration, which strongly correlates with reduced insulin-stimulated glucose disposal in skeletal muscle.

In normal conditions, fatty acids are stored in the adipose tissue as triglycerides and released during fasting. During the postprandial state, blood glucose stimulates insulin secretion, which inhibits lipolysis in adipose tissue, therefore limiting the release of fatty acids. In insulin resistant individuals, the ability of insulin to inhibit lipolysis and reduce plasma fatty acid concentration is markedly impaired (Groop et al., 1991). This leads to a chronic activation of lipolysis and higher plasma fatty acid levels. Several studies have demonstrated that chronically elevated plasma fatty acid levels cause insulin resistance in skeletal muscle(Bajaj et al., 2005, Boden, 1997).

One of the proposed mechanisms to explain how fatty acids impair glucose oxidation in skeletal muscle was postulated by Randle and colleagues more than 40 years ago (Randle et al., 1963). They observed that incubation of rat heart with fatty acids was associated with an increase in intracellular concentrations of glucose-6-phosphate (G6P) and glucose. Moreover, incubation of diaphragm muscle with fatty acids led to an increase in glycogen accumulation. According to "Randle's Theory", fatty acid oxidation increases the ratios acetyl coenzyme A/coenzyme A and NADH/NAD+ in the mitochondria, leading to the inactivation of pyruvate dehydrogenase (PDH). Accumulation of citrate inhibits phosphofructokinase and increases intracellular concentrations of G6P, leading to activation of glycogen synthesis, inhibition of hexokinase II, increase in intracellular glucose content and, consequently, reduction in glucose uptake. Thus, this model is based on the inverse relationship between fatty acid availability and glucose utilization. Increase free fatty acid availability inhibits glucose utilization through inhibition of key enzymes involved in glucose metabolism.

In contrast with Randle's hypothesis, Roden and colleagues (Roden et al., 1996) demonstrated that a reduction in muscle glycogen synthesis by elevated fatty acids concentration occurred after a decrease in muscle glucose-6-phosphate levels. Thus, these results demonstrate that free fatty acids induce insulin resistance in humans by initial inhibition of glucose transport/phosphorylation, which is then followed by a reduction in both the rate of muscle glycogen synthesis and glucose oxidation. Therefore, according to Roden et al., insulin resistance induced by fatty acids is primarily associated with impaired glucose uptake rather than glucose accumulation (Roden et al., 1996).

To establish which of these two possible effects takes place, Dresner and colleagues (Dresner et al., 1999) measured intra-myocellular concentrations of free glucose in healthy people under conditions of high and low plasma fatty acids concentrations. If there was a block at the hexokinase step, as proposed by Randle, intra-myocellular glucose concentrations would be expected to increase. Instead, they noted that plasma fatty acid concentrations decreased the accumulation of intra-myocellular glucose, indicating that insulin-stimulated glucose transport activity was reduced. These results, like others carried out by Cline and colleagues (Cline et al., 1999), confirmed Roden's work and suggested that in people with T2DM impairment of insulin action in skeletal muscle is due to reductions in insulin-stimulated glucose transport rather than glucose accumulation.

A second mechanism proposed to explain the pathogenesis of skeletal muscle insulin resistance is related to endoplasmic reticulum (ER) stress (Hotamisligil, 2010). ER is an intracellular membranous network responsible for synthesis, folding, maturation, trafficking and targeting of secreted and transmembrane proteins. It also plays a critical role as a regulator of Ca^{+} homeostasis and lipid biosynthesis. In some diseases, protein synthesis increases in ER-lumen and proteins cannot fold correctly, affecting ER homeostasis. Impairment of ER homeostasis activates an elaborate adaptive stress response, known as "unfolded protein response" (UPR), and results in the phosphorylation and activation of JNK. The link between T2DM, insulin resistance and ER stress in skeletal muscle is still

unclear. It has been demonstrated that ER stress occurs *in vivo* in skeletal muscle when mice are fed a high fat diet (Deldicque et al., 2010a). In another study (Deldicque et al., 2010b), the same authors observed that subjects on high fat diet had increased lipid content and insulin resistance in skeletal muscle with no change in ER stress markers.

Inflammation has also been proposed as a potential mechanism involved in the development of impaired insulin sensitivity. Fatty acids activate inflammatory signals by promoting secretion of pro-inflammatory cytokines including TNFalpha, IL-1beta, and IL-6. Furthermore, fatty acids can directly interact with members of the Toll-like receptor (TLR) family, promoting activation of JNK and IKKbeta. This activation leads to degradation of the inhibitor of kappa beta (IKB) and Nuclear factor-kappa beta (NFKB) activation. This is associated with a decrease in insulin action due to the phosphorylation of IRS-1. A study carried out by Tsukumo and co-workers demonstrated that mice containing a loss of function mutation in the *tlr4* gene (toll-like receptor 4) were partially protected from lipid-induced muscle inflammation (Tsukumo et al., 2007), highlighting the importance of this receptor in skeletal muscle insulin sensitivity.

5. Biology of the mitochondria

Mitochondria are doubled-membrane organelles that constitute the major site for oxidative energy production in the cell. Mitochondria are the only mammalian organelles that contain extra-nuclear DNA (mtDNA), which encodes for 37 genes including 13 subunits of the electron transport chain (Kelly and Scarpulla, 2004). Besides generating the majority of cellular ATP via oxidative phosphorylation (OXPHOS), many other essential cellular functions take place in this organelle. Examples of these include the generation of numerous metabolites via the tricarboxylic acid (TCA) cycle, oxidative catabolism of amino acids and fatty acids, synthesis of ketone bodies, ornithine cycle (also known as the urea cycle), control of cytoplasmic reticulum and calcium signaling (Murgia et al., 2009, Rimessi et al., 2008), synthesis of cellular Fe/S clusters that are essential cofactors for protein translation and DNA repair (Lill and Muhlenhoff, 2008) and generation of reactive oxygen species (ROS) with important signalling functions (Starkov, 2008, Murphy, 2009) and potential damaging consequences.

5.1. Oxidative phosphorylation (OXPHOS)

Mitochondria are able to generate energy by oxido-reduction reactions and proton translocation derived from carbohydrates (TCA cycle), amino acids and fatty acids (β-oxidation). For this purpose, oxygen is consumed to generate water, heat and adenosine triphosphate (ATP). The inner membrane invaginations of the mitochondria, called cristae, contain all transmembrane proteins of the electron transfer system (ETS) and the ATP synthase (Benard and Rossignol, 2008, Vonck and Schafer, 2009). All components of the TCA cycle and β-oxidation pathway are located inside the mitochondrial matrix. Oxidation of substrates generates reduced nicotinamide adenine dinucleotide (NADH) and reduced flavin adenine dinucleotide (FADH2) that will provide electrons to the ETS. Four different complexes –named from complex I to complex IV- form the ETS. Electrons flow from donors

(NADH at complex I and FADH2 at complex II) to an oxygen molecule forming H_2O at complex IV. There is a parallel translocation of protons to the intermembrane space from the matrix that creates an electrochemical gradient used by ATP synthase in a coupled manner to generate ATP. This electrochemical gradient can also dissipate through uncoupling proteins (UCPs) using a non-ATPase-coupled proton leak and generating heat in a process called thermogenesis. The high electronegative potential generated can also drive the entry of calcium into the matrix. Another phenomenon is the loss of electrons during the ETS that can result in generation of reactive oxygen species.

5.2. Mitochondrial biogenesis and dynamics

Mitochondrial biogenesis is defined as the generation of more mitochondrial mass and takes place in response to increased energy demand. These organelles have a heterogeneous morphology due to its dynamic nature. Mitochondrial dynamics is a relatively novel concept that includes movement of mitochondria along the cytoskeleton, regulation of mitochondrial architecture (morphology and distribution), and connectivity mediated by tethering and fusion/fission events (Liesa et al., 2009). Mitochondrial fusion/fission events allow the transcriptional products of mtDNA along with multiple metabolites to be shared within the mitochondrial reticulum. It has been recently established that mitochondrial fission and fusion contribute to multiple essential functions including calcium handling, ROS production and energy output (Chen and Chan, 2005, Parone et al., 2008, Soubannier and McBride, 2009). The relevance of these events in mitochondrial and cell physiology has been partially unraveled and observed that the disruption of such processes results in mitochondrial heterogeneity and dysfunction (Zorzano et al., 2009, Chan, 2006). Therefore, a fine-tune regulation of mitochondrial biogenesis and dynamics is necessary to obtain and maintain functional mitochondria.

Mitochondrial biogenesis is a complex process that requires the expression of a large number of proteins encoded by both nuclear and mitochondrial genomes. The mitochondrial genome encodes only 13 proteins, which are essential subunits of the respiratory complexes. This genome also provides the 22 tRNAs and 2 rRNAs necessary for the translation of these mitochondrial-encoded proteins. In contrast, transcription of the mitochondrial genome is encoded by the nuclear genome, which is under the control of a single transcription factor named TFAM. Other components needed for the transcription of the mitochondrial genome, including POLRMT, TFB1M, TFB2M, and mTERF are also encoded by nuclear genes. Therefore, fine-tuned coordination is required between the mitochondrial and the nuclear genomes to orchestrate the expression of proteins necessary for a successful mitochondrial biogenesis. This coordination is achieved by complex regulatory mechanisms that involve the action of a relatively small number of nuclear transcription factors, which are discussed in detail below. These transcription factors are in turn regulated by cofactors that integrate physiological signals with the activity of the transcription factors to regulate mitochondrial biogenesis in response to environmental stimuli. Among the most important cofactors are the PGC-1 coactivator family members, which are also discussed in more detail below (Kelly and Scarpulla, 2004).

5.3. Nuclear transcription factors involved in mitochondrial biogenesis

Through their DNA-binding domain, transcription factors bind to specific sequences in the gene promoter region to regulate transcription of a subset of genes. Several transcription factors have been shown to regulate expression of genes involved in the respiratory chain and mitochondrial metabolism, however only a few are considered the major transcription factors crucial for mitochondrial biogenesis.

5.3.1. Nuclear Respiratory Factor 1 (NRF-1)

NRF-1 has a fundamental role in coordinating nuclear and mitochondrial transcription. It induces expression of TFAM, TFB1M and TFB2M (Virbasius and Scarpulla, 1994, Gleyzer et al., 2005), which are essential proteins for the transcription of the mitochondrial genome, and also TOMM20, a key protein required for the transport of nuclear-encoded proteins into the mitochondria. It has also been shown to regulate multiple subunits of the respiratory chain as well as other proteins involved in other mitochondrial functions. Disruption of the NRF-1 gene in mouse models results in mtDNA depletion and impaired mitochondrial membrane potential with an early embryonic lethal phenotype (Huo and Scarpulla, 2001).

5.3.2. Nuclear Respiratory Factor 2 (NRF-2/GABP)

A second nuclear respiratory factor was identified based on its ability to induce expression of a subunit of cytochrome c oxidase, COXIV, and was found to be a complex of the DNA-binding subunit alpha (GABPalpha) and four other subunits (beta1, beta2, gamma1, and gamma2). This respiratory factor was named NRF-2 and was subsequently identified as the human homolog of the mouse GABP (Virbasius et al., 1993). NRF-2 has been shown to regulate expression of key proteins involved in mitochondrial biogenesis and function, including TFAM, TFB and all cytochrome C oxidase isoforms (Gleyzer et al., 2005, Ongwijitwat and Wong-Riley, 2005, Virbasius et al., 1993). Similarly to NRF-1, disruption of the NRF-2 gene also produces a lethal phenotype (Ristevski et al., 2004).

5.3.3. Estrogen-Related Receptor alpha (ERRalpha)

ERRalpha mediates expression of a wide range of genes, including those responsible for fatty acid uptake and oxidation as well as genes for oxidative phosphorylation (Mootha et al., 2004, Huss et al., 2002). Although structurally related to the estrogen receptor, ERRalpha does not bind estrogen. Instead, it is a member of a family of orphan nuclear receptors that also include ERRbeta and ERRgamma. Unlike NRF-1 and NRF-2 where gene knockout proves lethal, disruption of ERRalpha results in a viable phenotype showing decreased body weight and adipose depot size (Luo et al., 2003). This mouse shows normal energy expenditure with no major decrease in mitochondrial proteins. This can be explained by compensation by the other members of the transcription factor family.

5.3.4. Other transcription factors

While not directly involved in transcription of mitochondrial biogenesis or respiratory chain genes, other transcription factors including PPARalpha, PPARdelta, and YY1 are also important for providing other mitochondrial proteins. PPARalpha is responsible for expression of lipid metabolism and mitochondrial fatty acid oxidation genes (Lee et al., 1995, Leone et al., 1999). Through inducible tissue-specific loss of function knockout mouse models, PPARdelta has been shown to regulate mitochondrial biogenesis in skeletal muscle and heart (Schuler et al., 2006, Wang et al., 2010). Finally, YY1 has been shown to activate cytochrome c expression (Seelan and Grossman, 1997, Basu et al., 1997) as well as several key genes for mitochondrial respiration (Cunningham et al., 2007).

5.3.5. PGC-1 coactivator family

While the transcription factors discussed above are part of the transcriptional machinery necessary for mitochondrial biogenesis, the members of the PGC-1 coactivator family provide the integration of physiological stimuli with the transcription factors to adapt mitochondrial biogenesis to changes in the environment. PGC-1 coactivators lack a DNA-binding domain, but they are able to interact with and activate several transcription factors by recruiting other cofactors with chromatin-remodeling activities (Monsalve et al., 2000). PPARgamma coactivator 1alpha (PGC-1alpha), the founding member of the PGC-1 family, was first identified by its ability to activate PPARgamma in brown adipocytes (Puigserver et al., 1998). PGC-1beta and PRC where subsequently identified based on their structural similarity with PGC-1alpha (Lin et al., 2002a, Andersson and Scarpulla, 2001). Interestingly, NRF-1, NRF-2, ERRalpha, YY1 and the PPAR family members are among the transcription factors the PGC-1 family members are able to coactivate (Wu et al., 1999, Cunningham et al., 2007), underlying the importance of these coactivators in the regulation of mitochondrial biogenesis. This role of PGC-1alpha and PGC-1beta in mitochondrial gene expression is well documented in gain of function experiments, where increased expression of these coactivators in skeletal muscle results in an induction of a wide array of genes involved in mitochondrial biogenesis and function (Wu et al., 1999, Lin et al., 2002b, Arany et al., 2007). Furthermore, muscle-specific disruption of PGC-1alpha gene in mice shows decreased expression of mitochondrial genes, resulting in a switch from oxidative fibers to more glycolytic fibers, impairing their endurance capacity (Handschin et al., 2007).

PGC-1alpha is highly regulated at both the transcriptional level and post-translational level, primarily through phosphorylation (Jäger et al., 2007, Li et al., 2007, Rodgers et al., 2010) and acetylation (Lerin et al., 2006, Rodgers et al., 2005). It is this regulatory capacity that allows PGC-1alpha to respond to physiological stimuli and activate the mechanisms leading to increased mitochondrial biogenesis. PGC-1alpha activity is determined by its acetylation status, regulated by the balance between acetylation mainly by the histone acetyltransferase GCN5 (Lerin et al., 2006) and deacetylation largely by the NAD+ dependent deacetylase SIRT1 (Rodgers et al., 2005). This acetylation/deacetylation regulatory mechanism is

involved in the integration of nutrient sensing with transcriptional regulation of mitochondrial genes in skeletal muscle (Gerhart-Hines et al., 2007). In this context, caloric restriction has been proposed to increase mitochondrial biogenesis at least in part through activating SIRT1 and inducing PGC-1alpha deacetylation increasing its transcriptional activity (Baur et al., 2006, Lagouge et al., 2006). Physical exercise has also been recognized as a main activator of mitochondrial biogenesis. In the muscle cell, the AMP-dependent protein kinase (AMPK) responds to low energy levels (increase in AMP content) by inducing a signaling cascade that results in the activation of catabolic pathways and inhibition of anabolic pathways in an attempt to restore energy levels. Therefore, AMPK has been recognized as a key mediator in the physiological and metabolic adaptation to physical exercise. Interestingly, AMPK can directly phosphorylate PGC-1alpha and activate its transcriptional activity regulating expression of mitochondrial genes (Jäger et al., 2007). Furthermore, it has been recently shown that AMPK activation results in a net increase of NAD+ levels with the consequent induction of SIRT1 activity and PGC-1alpha deacetylation (Canto et al., 2010).

6. Mitochondrial dysfunction as a potential mechanism underlying skeletal muscle insulin resistance

Mitochondrial adaptations (biogenesis and dynamics) and function largely affect muscle metabolism and have a significant impact on whole-body metabolism (Patti et al., 2010). As mentioned before, metabolic flexibility is defined as the ability to rapidly modulate substrate oxidation as a function of environmental, hormonal and different energy conditions (Storlien et al., 2004). Defects in pathways controlling glucose and energy homeostasis in skeletal muscle have been shown to impair these adaptations, leading to metabolic inflexibility. What it is important for the role played by mitochondrial dysfunction in T2DM etiology is that this state of metabolic inflexibility is a hallmark of the development of skeletal muscle insulin resistance (Storlien et al., 2004).

Mitochondrial dysfunction is a term that could imply several definitions due to the multiple functions that take place in this organelle. For the purposes of this chapter, we will define mitochondrial dysfunction as both the reduction in mitochondrial oxidative activity and in mitochondrial adenosine triphosphate (ATP) synthesis. Although mitochondrial dysfunction is related to a broad range of diseases, in this chapter we will focus on mitochondrial respiratory dysfunction related to muscle insulin resistance and T2DM etiology.

6.1. Early evidences relating insulin resistance and skeletal muscle mitochondrial dysfunction

Several key studies published between 1999 and 2005 laid the foundation for understanding the underlying mechanisms between mitochondrial dysfunction and subsequent insulin resistance in skeletal muscle and development of T2DM. Significant results from these studies are summarized below.

6.1.1. Dysregulation of skeletal muscle fat oxidation in obesity

The first studies that identified a relationship between alterations in muscle metabolism and insulin resistance did not mention any link with mitochondrial dysfunction (Kelley et al., 1999). However, research performed by Kelley and co-workers addressed why the pattern of fatty acid utilization in skeletal muscle during fasting conditions might be associated with obesity-related insulin resistance, which is relevant for the scope of this chapter. The study included 16 lean and 40 obese volunteers with leg balance measurements of glucose and free fatty acid uptake. Indirect calorimetry across the leg was also measured in order to determine substrate oxidation during fasting and insulin-stimulated conditions. This study demonstrated that fatty acids were the predominant substrate oxidized by skeletal muscle during fasting conditions in lean subjects. However, rates of fatty acid oxidation during fasting were significantly lower in obese subjects, even though rates of fatty acid uptake were similar to those of lean subjects. Furthermore, the respiratory quotient values across the leg showed a reduced reliance on lipid oxidation in obese subjects. What it is also important is that weight loss only partially improved these patterns; the leg respiratory quotient in obese subjects was unchanged between pre- and post-weight loss, so the reliance of skeletal muscle in fat oxidation during fasting conditions was not improved. The authors suggested that their data pointed to these defects as primary impairments leading to obesity, rather than resulting from obesity. Based on these data and previous observations from the same group (Kelley et al., 1993, Kelley et al., 1999) it could be concluded that the elevated intra-myocellular lipid accumulation in skeletal muscle of obese subjects derives from a reduced capacity for fatty acid oxidation, and this inflexibility in regulating fatty acid oxidation rates, more than fatty acid uptake itself, is related to insulin resistance.

6.1.2. Muscle mitochondria in obesity and type 2 diabetes

In this study, Kelley and co-workers provided early evidence that mitochondrial dysfunction in human skeletal muscle contributes to the development of insulin resistance and progression to T2DM (Kelley et al., 2002b). Previous work by the same group demonstrated that the severity of skeletal muscle insulin resistance in T2DM and obesity is related to diminished activity of oxidative enzymes (Simoneau and Kelley, 1997). Furthermore, triglyceride accumulation in skeletal muscle is also correlated with the severity of insulin resistance and with diminished oxidative enzyme activity. Because it was known that skeletal muscle depends on oxidative phosphorylation to produce energy and that insulin resistance in T2DM and obesity involves altered oxidation of carbohydrates and lipids, the authors attempted to elucidate the potential contribution of mitochondrial dysfunction to skeletal muscle insulin resistance in humans. For this purpose *vastus lateralis* muscle samples from lean controls without T2DM, obese subjects with or without T2DM were obtained. An assessment of the activity of the mitochondrial OXPHOS system and a quantitative study of the mitochondria morphology by transmission electron microscopy was performed in the different muscle biopsies. Creatine kinase and citrate synthase activities were measured as markers of muscle fiber content and mitochondrial content, respectively. Results showed that skeletal muscle mitochondria structure and functional capacity were impaired in T2DM subjects and, to a lesser degree, in obese subjects.

Mitochondrial respiratory complex I activity was reduced by 40% in skeletal muscle from subjects with T2DM when compared to lean controls without diabetes. Moreover, skeletal muscle mitochondrial area and size were smaller in obesity and T2DM and, in some instances, particularly in T2DM, severely damaged. Although age can affect the size of mitochondria, in this case aging did not account for the ~30% reduction in size in obesity and T2DM.

Based on their results, authors proposed a potential mechanism that could explain how impaired mitochondrial function leads to insulin resistance in skeletal muscle, which would be lipid accumulation within myocytes. This was not a new finding, as previous studies (Kelley et al., 2002a) from the same group had shown that increased lipid accumulation in skeletal muscle is associated with insulin resistance and, in turn, lipid accumulation in skeletal muscle in obesity and T2DM is related to a reduced oxidative enzyme activity. Therefore, based on their findings, the authors stated that impaired mitochondrial functional capacity in skeletal muscle can lead to insulin resistance and further T2DM.

6.1.3. Downregulation of oxidative metabolism genes in humans with insulin resistance and diabetes

Patti and colleagues addressed how gene regulation was modulated by T2DM (Patti et al., 2003). High-density oligonucleotide arrays were performed using mRNA samples from skeletal muscle of people with or without T2DM and with prediabetes (insulin-resistant subjects at high risk for T2DM) to identify genes differentially expressed. The results showed that skeletal muscle from subjects with prediabetes and T2DM had decreased expression of oxidative phosphorylation genes, many of which are regulated by nuclear respiratory factor (NRF)-dependent transcription. A decreased expression of the co-activators PGC-1alpha and PGC-1beta, both of which induce NRF-dependent transcription, was also found. Therefore, subjects with insulin resistance and T2DM have a reduced expression of multiple (NRF-1)-dependent genes encoding key enzymes in oxidative metabolism and mitochondrial function. It seems that PGC-1 expression may be responsible for decreased expression of NRF-dependent genes due to alterations in the primary sequence and environmental risk factors for T2DM such as aging, fiber type composition, insulin resistance itself and inactivity, subsequently leading to the metabolic disturbances characteristic of insulin resistance and T2DM.

6.1.4. PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes

This study extended the results obtained by Patti and colleagues (Patti et al., 2003). Mootha and co-workers used Gene Set Enrichment Analysis, designed to detect modest but coordinated changes in the expression of groups of functionally related genes to study differential expression among healthy individuals, impaired glucose tolerance subjects, and subjects with T2DM (Mootha et al., 2003). They named OXPHOS-CR to a subset of genes, which include about two-thirds of the OXPHOS genes, strongly expressed in skeletal muscle,

heart and brown adipose tissue. No relationship was found between body mass index (BMI) or waist-to-hip ratio and OXPHOS-CR expression, and neither between quantitative measures of fiber types and OXPHOS-CR. However, expression of OXPHOS-CR correlated positively with the aerobic capacity of the individuals under study and negatively to diabetes. In summary, a set of genes involved in oxidative phosphorylation, whose expression was coordinately decreased in skeletal muscle of T2DM subjects, were identified. Thus, authors hypothesized that the decreased expression of OXPHOS-CR genes might contribute to T2DM. Expression of this gene set was induced by PGC-1alpha, which expression is higher in tissues of insulin-mediated glucose disposal and correlated with total body aerobic capacity.

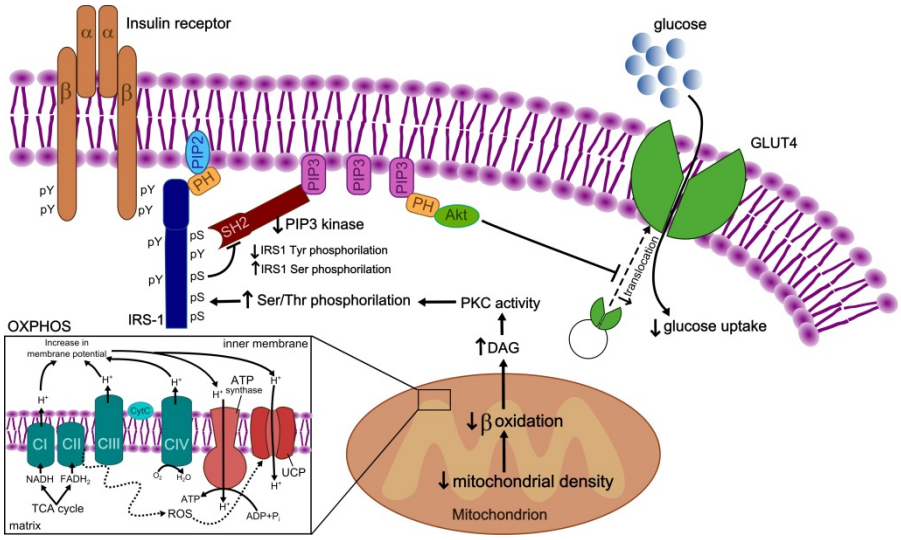
6.1.5. Impaired mitochondrial activity in insulin-resistant offspring of subjects with T2DM

In this study Petersen and co-workers aimed to determine the potential mechanism for the intra-myocellular accumulation of lipids leading to insulin resistance (Petersen et al., 2004). As previously stated, increases in intra-myocellular triglyceride content can occur as a result of increased delivery of fatty acids from lipolysis and/or decreased rates of mitochondrial oxidative phosphorylation. Young and lean insulin-resistant offspring of subjects with T2DM and insulin-sensitive subjects were studied. To test their hypotheses, authors utilized hyperinsulinemic-euglycemic clamps in these subjects to measured intra-myocellular lipid and intrahepatic triglyceride content, assessed whole-body and subcutaneous fat lipolysis rates and determined mitochondrial oxidative-phosphorylation activity in muscle by magnetic resonance spectroscopy. The insulin-stimulated rate of glucose uptake was 60% lower in the insulin-resistant subjects, which could be explained by a 70% reduction in insulin-stimulated non-oxidative muscle glucose metabolism. They also observed an 80% increase in intra-myocellular lipid content and a 30% reduction in mitochondrial oxidative phosphorylation, suggesting that subjects with T2DM have an inherited reduction in mitochondrial content in muscle, which in turn may be responsible for the reduced rates of mitochondrial oxidative phosphorylation. In summary, their data supports the hypothesis that insulin resistance in skeletal muscle of insulin-resistant offspring of subjects with T2DM is linked to an impairment of fatty acid metabolism, potentially due to an inherited defect in mitochondrial oxidative phosphorylation (Petersen et al., 2004).

6.2. Putting the pieces together, the link between mitochondrial dysfunction and T2DM

In the year 2005 Drs. Lowell and Shulman wrote a viewpoint where they hypothesized that insulin resistance and hyperglycemia could be caused by a primary mitochondrial dysfunction (Lowell and Shulman, 2005). Insulin resistance occurs due to the accumulation of intracellular fatty acyl CoA and diacylglycerol, which in turn activate critical signal transduction pathways, leading to suppression of the insulin signaling pathway. Among the metabolic perturbations that caused accumulation of fatty acids in liver and/or skeletal muscle and defects in the ability of these organs to metabolize fatty acids, mitochondrial dysfunction seemed to be the reason for inducing insulin resistance. The suggested

hypothesis was that insulin resistance in humans arises from defects in mitochondrial fatty acid oxidation, which in turn lead to increases in intracellular fatty acid metabolites such as fatty acyl CoA and diacylglycerol that disrupt insulin signaling pathway (Figure 2).



OXPHOS, oxidative phosphorylation; CI-CII-CIII-CIV complexes I, II, III and IV within the oxidative phosphorylation system; CytC, cytocrome C; TCA, tricarboxylic acid cycle;NADH, nicotinamide adenine dinucleotide; FADH2, flavin adenine dinucleotide (hydroquinone form); ATP, adenosine triphosphate; ADP, adenosine diphosphate; Pi, inorganic phosphate; DAG, diacylglycerol; Thr, threonine; Ser,S, serine; Tyr,Y, tyrosine; IRS-1, insulin receptor substrate 1; PIP2, phosphatidyil inositol 2; PH, pleckstrin homology domain of the IRS-1; PIP3, phosphatidyl inositol 3; SH2, Src homology 2 domain; Akt, also known as protein kinase B; GLUT4, glucose transported type 4.

Figure 2. Fat-induced insulin resistance hypothesis in skeletal muscle.

T2DM and obesity characterize by chronic nutrient oversupply that can lead to an increase of reducing equivalents (NADH and FADH2) which increases the flux through the oxidative respiratory system (OXPHOS) in the inner membrane in the mitochondria and, in the absence of increased ATP demand, results in ROS generation creating oxidative stress. ROS increased concentration would lead to a diminished mitochondrial density/content or an inherited condition that in turn lead to a mitochondrial dysfunction and consequent decrease in beta-oxidation increasing the intra-myocellular long-chain fatty acids and diacylglycerol (DAG) concentrations. DAG would then activate the phosphorylation of serines and threonines of the insulin receptor susbtrate 1 (IRS-1) through enzymes such as protein kinases C (PKC). PKCs activate the serine kinase cascade and increase the IRS-1 serine (Ser, S) phosphorylation of the the insulin receptor susbtrate 1 (IRS-1). The phosphorylation of serines located in critical sites leads to a blockage of the IRS-1 tyrosines (Tyr, Y) phosphorylation by the insulin receptor, inhibiting insulin-induced phosphatidyl inositol 3-kinase activity (PI3-kinase) resulting in a decreased insulin-stimulated Akt activity. Akt reduced activity fails to activate the translocation of GLUT4 to the membrane, diminishing the insulin-induced glucose uptake and impairing the removing of glucose from blood.

It is still uncertain whether skeletal muscle mitochondrial dysfunction is a cause or rather a consequence of the metabolic derangements that contribute to insulin resistance in T2DM, including lipid accummulation, pro-inflammatory signals or endoplasmic reticulum stress. However, given its complex pathophysiology, establishing causality has proved difficult and the mechanisms leading to insulin resistance remain elusive.

7. Current status of the topic

It is well established that mice and rats with a chronic exposure to a high fat diet (HFD) develop obesity, insulin resistance and, in the long term, T2DM (Oakes et al., 1997, Surwit et al., 1988). Furthermore, it has been shown that skeletal muscle mitochondrial content is diminished in T2DM individuals (Patti et al., 2003, Kelley et al., 2002b). These features, together with impaired energy substrate utilization and the observation that these deleterious effects are not restricted to skeletal muscle, led to the hypothesis that mitochondrial dysfunction plays a major role in T2DM etiology (Lowell and Shulman, 2005). Since the publication of this hypothesis, there has been a growing interest in further assessing the potential implication of mitochondrial function in the etiology of this metabolic disease. One of the first attempts to clearly prove this hypothesis used transgenic mice with defective mitochondria in order to observe whether they would develop T2DM (Pospisilik et al., 2007). Earlier reports from this same group have shown that conditional deletion of apoptosis inducing factor (AIF) provokes OXPHOS dysfunction (Vahsen et al., 2004). Initially, AIF was considered as a mitochondrial protein involved in signaling events leading to cell death. Subsequent studies have demonstrated that the primary physiological role of AIF is the maintenance of an efficient mitochondrial respiratory system. Studies assessing whole body glucose homeostasis and diet-induced obesity and diabetes either in tissue specific (liver and skeletal muscle) AIF knockout mice or in mice with ubiquitous OXPHOS defects showed that these mice were more insulin sensitive and were protected against diet-induced obesity and diabetes, in contrast with previous hypotheses (Pospisilik et al., 2007). Recently, this observation has been confirmed in another study using rats fed with an iron-deficient diet, which provokes a reduction in the iron containing proteins of OXPHOS (Han et al., 2011). Thus, rats under a high fat and iron-deficient diet are protected against high fat diet-induced insulin resistance in skeletal muscle despite a lower fatty acid oxidation capacity (Han et al., 2011).

7.1. Controversy about the effects of high fat diet feeding in skeletal muscle oxidative capacity

In 2007, two different studies were published addressing whether a high fat diet (HFD) decreases or improves skeletal muscle mitochondrial oxidative capacity. In one (Garcia-Roves et al., 2007), rats were fed with a HFD during 4 weeks in order to raise circulating fatty acids and therefore to study the mechanisms that regulate the already reported improved fatty acid oxidation capacity of glycolytic skeletal muscle. Rats fed with the HFD regime showed higher fatty acids content, increased skeletal muscle fatty acid oxidative

capacity in the epitrochlearis (glycolytic muscle), increased expression of enzymes of the fatty acid oxidation pathway and increased protein content of carboxylic acid cycle and OXPHOS system markers. Furthermore, this study showed that this metabolic adaptation occurs through activation of the peroxisome proliferated activated receptor delta (PPARdelta), a nuclear receptor responsible for regulating transcription of enzymes that belong to the fatty acid oxidation pathway and mitochondrial biogenesis process. Fatty acids, mostly unsaturated, are ligands and activators of PPARs, which explain the metabolic regulations observed in this study (Garcia-Roves et al., 2007). Similar results were published, almost simultaneously, by Cooney and colleagues in mice (Turner et al., 2007). C57BL/6J mice were on a HFD either for 5 or 20 weeks. In both periods of time HFD mice showed an increased capacity to oxidize fatty acid in skeletal muscle, concomitantly with an increased enzymatic activity of key proteins in the fatty acid oxidation pathway and higher protein content of different mitochondrial markers. Most importantly, these improvements in fatty acid handling and mitochondrial respiration in fat-fed mice occurred at the time these animals showed skeletal muscle insulin resistance and impaired whole body glucose handling (Turner et al., 2007). These observations were corroborated in the same study using a rat model of obesity (Zucker rats on a HFD) or a mouse model of diabetes and obesity, the db/db mouse (Turner et al., 2007). One year later Holloszy's laboratory, in a follow-up of Garcia-Roves' study, showed that rats fed with two different high fat diets (50% of energy from flax seed/olive oil or lard/corn oil) had skeletal muscle insulin resistance with improved mitochondrial content (Hancock et al., 2008). Therefore, results from these studies led researchers to question whether mitochondrial dysfunction plays a major role in the etiology of T2DM. It seems more probable that skeletal muscle insulin resistance per se or skeletal muscle inactivity could be the origin of the observed decrease in mitochondrial content in subjects with T2DM.

As described over these lines it is uncertain whether skeletal muscle mitochondrial dysfunction plays a critical role in the development of T2DM. We continue the chapter presenting the more recent studies on this topic. First, we will address human based studies and afterwards recent animal studies that provide some relevant information into the field.

7.2. Human studies

It is important to note that in skeletal muscle two different mitochondrial populations exist: subsarcolemmal and intermyofibrillar. Subsarcolemmal mitochondria are located beneath the sarcolemma and have a lower oxidative rate than intermyofibrilar mitochondria. Furthermore, both mitochondrial populations play different roles in skeletal muscle metabolism and, therefore, respond differently to physiological or pathophysiological situations (Palmer et al., 1977). Kelley and coworkers compared both skeletal muscle mitochondrial subpopulations in lean, obese, and T2DM subjects (Ritov et al., 2005). The main observation of this study was that the electron transfer system was dramatically reduced in the subsarcolemmal mitochondrial population in obese subjects, and even to a higher degree in T2DM subjects when compared to lean controls. However, physical activity level was not controlled, therefore it is unknown whether differences in mitochondrial

activity could be related to this parameter. Thus, the authors concluded that an impaired subsarcolemmal mitochondrial activity may have a critical role in the pathogenesis of insulin resistance in T2DM (Ritov et al., 2005).

Similar conclusions are obtained in a study carried out by Schrauwen-Hinderling and colleagues (Schrauwen-Hinderling et al., 2007) where they attempted to distinguish between the relevance of intra-myocellular lipid content and mitochondrial dysfunction in skeletal muscle insulin resistance. For this purpose, they compared overweight subjects with high intra-myocellular lipid content to weight-matched T2DM subjects. The study showed that T2DM subjects had impaired mitochondrial function and similar levels of intra-myocellular lipid content when compared to weight-matched controls. These data provide more evidence supporting the link between mitochondrial dysfunction and T2DM, although they do not imply causality (Schrauwen-Hinderling et al, 2007). Similar results were found in a subsequent study where Ritov and colleagues compared mitochondrial function in lean, obese and obese-T2DM subjects (Ritov et al., 2010). Along with this idea, Shulman and co-workers performed a study on insulin resistant offspring of T2DM subjects. Offspring subjects with insulin resistance showed a decreased oxidative phosphorylation capacity and increased intra-myocellular lipid content. These results led the authors to conclude that a limited mitochondrial fatty acid oxidative capacity leads to an increased lipid accumulation and subsequently to skeletal muscle insulin resistance. However, the mechanism by which skeletal muscle intralipid accumulation leads to insulin resistance needs still to be elucidated (Befroy et al, 2007). Conversely, in another study performed in subjects with and without T2DM (Asmann et al., 2006), mitochondrial ATP production rate under controlled insulin and glucose levels at the post-absorptive state (low glucose, low insulin levels) and post-pandrial state (high glucose, high insulin levels) was evaluated. The authors found no major differences between subjects with and without T2DM at the post-absortive state. A lower response in the mitochondrial ATP production rate when insulin and glucose rose to achieve post-pandrial state values was observed in T2DM subjects. This study showed how skeletal muscle mitochondrial defects are not intrinsic in subjects with T2DM but are related to impaired insulin action (Asmann et al., 2006). Thus, it is difficult to discern whether mitochondrial dysfunction is a cause or rather a consequence of skeletal muscle insulin resistance due to the cross-sectional nature of most of the human studies carried out.

In 2007 Dela and co-workers added some relevant information to the field using high resolution respirometry to compare skeletal muscle mitochondrial function between T2DM and control subjects (Boushel et al., 2007). T2DM subjects showed a reduced OXPHOS and electron transport capacity when compared to control individuals, as previously observed. However, when the respiratory parameters were normalized by mitochondrial content the authors observed that T2DM subjects did not show mitochondrial dysfunction (Boushel et al., 2007). Therefore, skeletal muscle respiratory capacity could be diminished in T2DM subjects due to a reduction in mitochondrial content and not due to specific defects in the respiratory phosphorylation system. As mentioned before, the observation of a reduced mitochondrial content could be due to a lack of physical activity rather than to a genetic defect in these insulin resistant individuals. These results, showing that T2DM subjects have

similar mitochondrial respiratory capacity when normalized by mitochondrial content, were corroborated by a later study performed in lean and obese insulin resistant subjects by Mandarino and co-workers (Lefort et al., 2010). When mitochondrial function was assessed in isolated skeletal muscle mitochondria from lean and obese-insulin resistant subjects a similar maximal respiration rate was observed in both groups. However, obese-insulin resistant subjects showed a higher reactive oxygen species (ROS) production, which could influence insulin signaling. Additional information was obtained with the comparative analysis of mitochondrial proteins from lean and obese insulin resistant subjects by high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry pointing out to a lower content in obese-insulin resistant subjects for some of the complex I subunits, less carnitine palmitoyltransferase 1B (key enzyme for fatty acid oxidation) and lower content of enzymes involved in branched amino acid metabolism. These differences in skeletal muscle mitochondrial proteins from lean and obese-insulin resistant subjects could explain why there is a higher intra-myocellular lipid accumulation in insulin resistant muscles, a higher ROS production and an improper branched amino acid oxidation (Lefort et al., 2010). Another issue that had not been previously addressed was mitochondrial sensitivity (mitochondrial respiratory performance under submaximal concentrations of substrates). This point was addressed by Dela and co-workers in T2DM subjects, obese controls and age-matched lean individuals. T2DM subjects showed enhanced mitochondrial substrate sensitivity when compared to obese controls. This better mitochondrial substrate handling is limited to non-lipid substrates (malate, glutamate, and succinate) and it is unrelated with maximal respiratory capacity, due to the fact that maximal oxidation rates were the same for the different substrates and experimental groups under assessment (Larsen et al., 2011).

More information, discounting the hypothesis linking mitochondrial dysfunction with the pathogenesis of insulin resistance and subsequently T2DM, arrives from studies of low birth weight (LBW) individuals. It has been previously established that LBW is a risk factor of insulin resistance and T2DM (Phillips, 1998). A recent study by Brons et al. showed that young lean LBW subjects had abnormal glucose metabolism when compared to normal birth weight (NBW) controls, but skeletal muscle mitochondrial ATP production (both in arms and legs) and expression of oxidative phosphorylation genes were similar between LBW and NBW subjects (Brons et al., 2008). More clear evidence against the theory that mitochondrial dysfunction leads to insulin resistance and T2DM was supplied in a follow-up study (Brons et al., 2012). Brons and co-workers assessed skeletal muscle mitochondrial function in LBW and NBW subjects before and after 5 days of high fat diet overfeeding. LBW subjects developed peripheral insulin resistance after high fat diet overfeeding but without any detrimental effect on mitochondrial oxidative capacity (Brons et al., 2012).

7.2.1. Differences in between skeletal muscles, aerobic capacity and aging

The comparison of mitochondrial function in arm and leg skeletal muscles from obese control and T2DM subjects supplies additional and relevant information to determine whether mitochondrial dysfunction is implicated in T2DM etiology. Mitochondrial

respiration, when quantified per milligram of wet tissue or citrate synthase activity, was similar in permeabilized muscle fibers from deltoids muscle biopsies of T2DM and obese-control subjects. However, when mitochondrial respiration was measured from the *vastus lateralis* muscle a significant reduction was observed in T2DM subjects, as previously reported in other studies (Rabol et al., 2010). These results highlight the importance of assessing the physical fitness of the individuals, as this factor could explain the differences found in leg muscles oxidative capacity when healthy individuals and T2DM subjects are compared. The finding that muscle respiratory capacity is not impaired in arm muscles of T2DM subjects goes against the hypothesis of mitochondrial dysfunction playing a role in the pathogenesis of T2DM. More insight into this question was obtained in studies on aging, both in skeletal muscle insulin sensitivity and mitochondrial function. Karakelides and co-workers performed a study assessing insulin sensitivity and skeletal muscle mitochondrial function using a hyperinsulinaemic-euglycemic clamp technique and quantification of mitochondrial ATP production rate, respectively (Karakelides et al., 2010). The subjects were divided in four different groups: young lean, young obese, old lean, and old obese individuals and stratified by sex. The results obtained by this experimental approach indicated that insulin sensitivity is related to the levels of adiposity and not to aging. In contrast, skeletal muscle mitochondrial function is reduced by age and not related to the levels of adiposity. Furthermore, men showed a higher mitochondrial ATP production rate than women, but women were more insulin sensitive than men. Taken together, this study clearly dissociated skeletal muscle mitochondrial function from insulin sensitivity (Karakelides et al., 2010). However, the subjects were not matched by maximal oxygen uptake capacity (VO2max.), which could be a confounding factor due to the fact that aerobic capacity is related to mitochondrial respiratory capacity. A recent study addressed whether aging has an effect in mitochondrial function when VO2max. values are taken into account. Young and middle-aged individuals with matched VO2max. had similar skeletal muscle mitochondrial respiratory capacity per wet tissue weight. However, when mitochondrial respiratory capacity was normalized by mitochondrial content, middle-aged subjects showed a lower capacity per mitochondrial unit (assessed by mtDNA content), implying that with age skeletal muscle keeps mitochondrial respiratory capacity increasing mitochondrial density (Larsen et al., 2012).

7.2.2. Ethnicity and skeletal muscle insulin resistance

It is known that Asian-Indians have one of the highest prevalence of T2DM in the world. In order to understand the metabolic differences that lead to this higher prevalence, a study was performed by Nair and co-workers comparing diabetic Asian-Indians, non-diabetic Asian-Indians, and non-diabetic Northern European-Americans (Nair et al., 2008). A hyperinsulinaemic-euglycemic clamp assessing insulin sensitivity and whole body glucose disposal, along with skeletal muscle maximal mitochondrial ATP production rate, protein content and mRNA expression information of different mitochondrial markers was used to evaluate mitochondrial function in the three experimental groups. Diabetic Asian-Indians and non-diabetic Asian-Indians had similar mitochondrial function even though they had

marked differences in insulin sensitivity, an observation that does not support mitochondrial dysfunction playing a role in T2DM development. Moreover, when the two ethnicities were compared, Asian-Indians had a higher OXPHOS capacity than Northern European-Americans even though Northern European-Americans were more insulin sensitive, providing additional data against a causative role of mitochondrial dysfunction in T2DM etiology (Nair et al., 2008).

7.2.3. Lifestyle interventions in type 2 diabetic individuals

It has been shown that lifestyle interventions that achieve significant body weight loss, reduction of body fat content, and improved aerobic capacity, are one of the most important tools, if not the best, in the prevention or amelioration of T2DM (Knowler et al., 2002). After completing an exercise training regime (cycling on a cycloergometer for 45 minutes, twice a week, for 10 weeks), T2DM subjects showed improved skeletal muscle lipid oxidation and mitochondrial oxidative capacity (Bordenave et al., 2008). However, this study did not address whether this significant improvement in mitochondrial function was related to enhanced glucose homeostasis, skeletal muscle insulin resistance, or both. Meex and co-workers attempted to elucidate whether an exercise training regime (12 weeks of aerobic exercise) could have a positive effect on skeletal muscle mitochondrial dysfunction, elevated intra-myocellular lipid accumulation, and insulin resistance, characteristic features for T2DM (Meex et al., 2010). After the completion of the exercise training intervention both overweight controls and T2DM subjects showed improved insulin-mediated glucose disposal, and a significant increase in mitochondrial respiratory capacity measured by ^{31}P-magnetic resonance spectroscopy (Meex et al., 2010) and in permeabilised muscle fibers by high resolution respirometry (Phielix et al., 2010). T2DM subjects recovered metabolic flexibility and showed an increase in intra-myocellular lipid content, in relation with what had been observed in trained individuals and in disagreement with the hypothesis that increased skeletal muscle lipid storage is related to insulin resistance and mitochondrial dysfunction (Meex et al., 2010). Similar observations were obtained by Hey-Mogensen and co-workers in obese subjects with and without T2DM (Hey-Mogensen et al., 2010). After 10 weeks of aerobic training both experimental groups showed significant improvement in mitochondrial respiratory capacity despite the observation of significant differences in insulin sensitivity between both groups (Hey-Mogensen et al., 2010). Obese subjects with T2DM showed increased reactive oxygen species (ROS) production (Hey-Mogensen et al., 2010), which is in line with the results of Lefort and co-workers in a similar group of subjects (Lefort et al., 2010). Obese T2DM subjects that follow a lifestyle intervention program combining caloric restriction (25% reduction of daily energy intake) with regular physical activity (30-40 min aerobic exercise per session) achieved an approximately 7% weight reduction after four months. These individuals improved skeletal muscle mitochondrial density, citrate synthase activity and insulin sensitivity. Thus, proving that lifestyle interventions have a beneficial effect in the treatment of T2DM (Toledo et al., 2007). A follow-up study from the same research group (Toledo et al., 2008) attempted to discern whether mitochondrial improvement after weight loss due to a lifestyle intervention is

related to the decrease in body weight or requires the improvement in aerobic fitness obtained by regular exercise. Both intervention groups (diet alone and diet plus exercise) achieved the same body weight loss (10%) and similar insulin sensitivity improvements. However, only the subjects under the diet plus exercise intervention program showed a significant enhancement in mitochondrial function (Toledo et al., 2008). This observation is pertinent because it distinguishes between insulin resistance improvements and skeletal muscle mitochondrial performance.

Human studies have produced clear evidence that T2DM is associated with skeletal muscle mitochondrial dysfunction, but have not supported the claim that mitochondrial dysfunction plays a major role in the development of the disease. It seems more likely that mitochondrial dysfunction could be a consequence of the general metabolic disarrangement originated in most cases by nutrient oversupply and insulin resistant status. As detailed before, skeletal muscle mitochondrial function is most probably modulated by the aerobic capacity of the individual, sex, age and ethnicity.

7.3. Animal studies

We have already mentioned that rats (Garcia-Roves et al., 2007, Hancock et al., 2008) and mice (Turner et al., 2007) develop insulin resistance with increased skeletal muscle mitochondrial content and fatty acid oxidative capacity after following a HFD for several weeks. These studies provided one of the first experimental evidences to show a discrepancy between mitochondrial dysfunction and T2DM etiology. However, at the same time a study was published by Lionetti and co-workers (Lionetti et al., 2007) in which rats were fed either with a low-fat diet or HFD for 7 weeks. The results obtained in this research showed how HFD induced hyperglycemia in rats and that it was related to an elevated fat supply, but not to higher energy uptake. Moreover, derangements in glucose handling are associated with a lower functionality of the subsarcolemmal mitochondrial population in skeletal muscle. This observation is in line with mitochondrial dysfunction playing a role in the development of T2DM and is also in agreement with a human study performed by Ritov and co-workers (Ritov et al., 2005).

To decipher the role played by skeletal muscle mitochondria in T2DM etiology, De Feyter and co-workers (De Feyter et al., 2008) compared obese ZDF rats to lean controls. These groups were studied at 6, 12 and 18 weeks of age, going from a pre-diabetic state (week 6) to T2DM (week 12 and 18). Mitochondrial function (measured *in vivo* by magnetic resonance spectroscopy and *in vitro* by enzymatic activities) together with intra-myocellular lipid content, fasting plasma glucose, insulin and free fatty acids data were collected. All these results showed how T2DM develops in line with increased intra-myocellular lipid accumulation but without a decrease in skeletal muscle mitochondrial function, again showing a clear divergence between T2DM and skeletal muscle mitochondrial function (De Feyter et al., 2008). These observations were corroborated in later studies by the same research group where mitochondrial respiratory capacity was measured by high-resolution respirometry (Lenaers et al., 2010). A similar analytical approach was also performed in mice fed with a HFD for 4

weeks (Bonnard et al., 2008) with similar results to those reported by De Feyter and co-workers (De Feyter et al., 2008), highlighting that mitochondrial dysfunction did not precede insulin resistance. However, when mice were fed with HFD for a longer term (16 weeks), mitochondrial defects were evident and mitochondrial respiration rates were lower in permeabilized muscle fibers. Mitochondrial defects were related to a higher ROS production rate and it was shown that a supplementation with antioxidants restored mitochondrial defects (Bonnard et al., 2008). Similar observations were made by Yokota and co-workers (Yokota et al., 2009) in mice fed with a HFD for 8 weeks. In Yokota's study they attempted to assess if insulin resistant mice with higher ROS production and impaired mitochondrial dysfunction would have a reduced exercise performance and whether antioxidant supplementation could reverse the adverse effect on exercise performance. When mice on a HFD were supplemented with apocynin, a compound that reduce free radical formation by inhibition of NAD(P)H oxidase activity (which reduces oxygen to superoxide), mice showed similar mitochondrial function, ROS production and exercise performance that mice under a low fat diet (LFD). However, these mice (HFD+apocynin), despite having better mitochondrial function, still showed impaired glucose tolerance (Yokota et al., 2009).

A subsequent study used Zucker rats to evaluate whether fatty acid transport played a key role in the observed increase in intra-myocellular fatty acid accumulation (Holloway et al., 2009). Additionally, fatty acid oxidation rates, mitochondrial density and intra-muscular triacylglycerol content were also determined. In summary, this study showed how skeletal muscle fatty acid transport was increased, mainly trough an upregulation of the levels of the fatty acid transporter CD36 (Holloway et al., 2009). Skeletal muscle subsarcolemmal mitochondria were increased and had a higher capacity to oxidize fatty acid, in contrast to previous observations (Ritov et al., 2005, Lionetti et al., 2007). However, based on their *in vitro* studies, fatty acid transport exceeded mitochondrial fatty acid oxidative capacity and therefore, intra-myocellular lipid accumulation was increased, which in a long term could explain skeletal muscle metabolic derangements observed in obesity and T2DM (Holloway et al., 2009).

Another aspect of lipid oversupply related to insulin resistance is the saturation of the phospholipids (fatty acid composition), which could alter membrane properties and therefore cellular function. Thus, insulin resistance correlated with the level of membrane phospholipids in skeletal muscle of Pima Indians, a population with the highest T2DM incidence (Pan et al., 1995). On this respect, two recent studies have addressed whether phospholipid fatty acid composition affects mitochondrial function and whether this is the mechanism leading to insulin resistance and further T2DM. For this purpose, C57BL/6J mice were fed with a LFD or a HFD for 3 or 28 days, and fasting glucose, insulin, gene expression profile, mitochondrial proteins and lipid composition were determined at each time point (3 and 28 days). After day 3, mice on a HFD showed increased fasting blood glucose and insulin levels. A marked increase in protein content of the different mitochondrial OXPHOS complexes was also observed at day 28 along with an increase in the degree of phospholipids saturation (de Wilde et al., 2008). Later on, the same research group attempted to understand whether mitochondrial membrane phospholipids composition

could affect mitochondrial function. In this study C57BL/6J mice were fed with a LFD or HFD for 8 or 20 weeks. From week 8, mice on a HFD had a higher level of saturation in their skeletal muscle mitochondrial membrane phospholipids. Mice were insulin resistant although mitochondrial respiratory capacity was not affected. Therefore, these observations were against mitochondrial dysfunction being a key feature in T2DM etiology and also questioning the role played by phospholipids composition in mitochondrial performance and glucose handling (Hoeks et al., 2011).

It seems clear, from the information obtained in the different studies using HFD-induced insulin resistance in mice or rats that at earlier and intermediate stages there are mitochondrial adaptations that improve skeletal muscle oxidative capacity to overcome fatty acid oversupply. To maintain skeletal muscle oxidative capacity, mitochondrial density is increased as showed by Van den Broek and co-workers (van den Broek et al., 2010) and in line with the observation made in humans by Dela and co-workers (Larsen et al., 2012).

7.4. Conclusions

Over these years we have reached a better understanding of how skeletal muscle mitochondria respond to different pathophysiological conditions. The evidence also clearly indicated that, at least at later stages in the etiology of the disease, mitochondrial dysfunction is present in T2DM both in humans and in animals. However, mitochondrial dysfunction does not play a major role in skeletal muscle insulin resistance and the development to T2DM. Most probably mitochondrial dysfunction results from the different disturbances produced by nutrient oversupply and lack of physical activity characteristic of the sedentary lifestyle of modern societies. Skeletal muscle mitochondrial function will be influenced by age, sex, ethnicity, and aerobic capacity. And more importantly, skeletal muscle mitochondrial defects and insulin resistance characteristic of T2DM could be rescue by changes in lifestyle that lead to an improved physical fitness and balanced diet.

7.5. Future perspectives

T2DM is a complex metabolic disease that involves disturbances not only in skeletal muscle, liver and pancreatic beta-cells (Defronzo, 1988) but also implies malfunction of adipose tissue, pancreatic alpha-cells, kidney, gastrointestinal tract and brain (Defronzo, 2009). Therefore, with the aim to find how to successfully prevent or treat T2DM we should address our research taking into account the systemic nature of this metabolic disease.

All cell types present in our body have a lower or higher degree of specialization that will determine their metabolic requirements and ability to adapt to different physiological or pathophysiological stresses. Thus, mitochondrial plasticity to metabolic derangements are cell-type specific. In this regard, Garcia-Roves and co-workers showed how different tissues respond differently to obesity and T2DM in terms of mitochondrial adaptive capacity (Holmstrom et al., 2012). Thus, db/db mice, an animal model of diabetes and obesity, showed clear evidence of mitochondrial dysfunction in liver at 16 weeks of age. However,

glycolytic skeletal muscles from the same mice had a better mitochondrial respiratory capacity and mitochondrial content than the lean control mice. More importantly, oxidative skeletal muscles show earlier evidences of mitochondrial function impairment. This study brings into account cell type plasticity and that time is also very important in the development of T2DM and should be taken into account to better interpret the different results already published (Holmstrom et al., 2012).

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Chapter 13

Nuclear-Mitochondrial Intergenomic Communication Disorders

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Additional information is available at the end of the chapter

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1. Introduction

Mitochondria are dynamic subcellular organelles present in virtually all eukaryotic cells with numerous functions. The most important of these functions is production of ATP; however they play an important role in various metabolic and developmental processes such as calcium homeostasis, apoptosis and programmed cell death, just to mention some. Mitochondria produce ATP by means of the mitochondrial respiratory chain (MRC) and oxidative phosphorylation (OXPHOS) system, a series of five enzyme complexes embedded in the inner mitochondrial membrane. Mitochondrial disorders most often refer to the dysfunction of OXPHOS system leading to deficiency in the ATP production. They are a group of genetically and phenotypically heterogeneous disorders with an incidence estimated to be between 1:5,000 and 1:10,000 live births [1].

MRC is the result of the interplay of two physically and functionally separated genomes, the nuclear DNA (nDNA) and mitochondrial DNA (mtDNA). Thirteen of the key structural polypeptides that constitute the multimeric subunits of the respiratory chain complexes are mtDNA encoded, in addition two ribosomal RNA (rRNA) and 22 transfer RNA (tRNA) that are required for initiating translation and protein synthesis [2]. Approximately 90 of the remaining proteins that make up the respiratory chain complexes are encoded by nDNA. Therefore, although human mtDNA encodes the basic machinery for protein synthesis, it depends entirely on the nucleus for the provision of enzymes for replication, repair, transcription, and translation. This dependency lies at the heart of several newly recognized human diseases that are characterized by secondary abnormalities of mtDNA.

The crosstalk between the two genomes is crucial for the cellular regulation of mtDNA integrity and copy number and correct mitochondrial protein production therefore mutations in genes involved in mitochondrial replication and maintenance can disrupt the

integrity of the mitochondrial genome, causing inter-genomic communication disorders. Multiple deletions, depletion of mtDNA or a combination of both phenomena (qualitative/quantitative lesions) in critical tissues, are the hallmarks of these disorders.

The focus of this chapter is to review the clinical and molecular etiologies of nuclear defects involved in mtDNA stability and in mitochondrial protein synthesis. The overview done here will hopefully provide insights towards best diagnostic strategies of mitochondrial cross–talk disorders, being useful for clinicians when facing similar cases. Additionally we will present a diagnostic algorithm for these diseases based on our knowledge.

2. Clinical manifestations of disorders affecting mtDNA integrity

Maintenance of mtDNA is controlled by an intricate homeostatic network, whose effectors are the various components of the mitochondrial replicosome and the many enzymes and carrier proteins that provide the mitochondrion with a balance supply of deoxyribonucleotides (Figure 1). As all of the factors are nDNA encoded, it is not surprising that mutations in genes involved in mitochondrial replication and maintenance can disrupt the integrity of the ''tiny'' mitochondrial genome [3] leading to multiple deletions or depletion [4]. The mitonuclear crosstalk has gained increased relevance in the past years and since then many genes have been identified as being involved in these diseases.

In the following section we will briefly review the clinical manifestations of both these group of disorders.

2.1. mtDNA multiple deletion syndromes

Mitochondrial diseases associated with the presence of multiple deletions of mtDNA are mostly autosomal dominant, occurring most often in adulthood. The size and terminals deletions are variable from one individual to another within the same family.

The main clinical manifestations associated with multiple deletions are:

i. **PEO** (autosomal dominant or recessive **P**rogressive **E**xternal **O**phthalmoplegia). The most common clinical features include adult-onset of weakness of the external eye muscles, bilateral ptosis, proximal muscle weakness wasting and exercise intolerance. Additional symptoms are variable, and may include cataracts, hearing loss, sensory axonal neuropathy, ataxia, depression, hypogonadism, and Parkinsonism. Less common features include mitral valve prolapse, cardiomyopathy, and gastrointestinal dysmotility. Both autosomal dominant and autosomal recessive inheritance can occur; autosomal recessive inheritance is usually more severe [5,6]. The multiple deletions associated with PEO are exclusively found in muscle tissues of patients.
ii. **SANDO** (**S**ensory **A**taxic **N**europathy, **D**ysarthria and **O**phthalmoparesis) is an autosomal recessive systemic disorder characterized mainly by adult onset of sensory ataxic neuropathy, dysarthria, and ophthalmoparesis. The phenotype varies widely, even within the same family, and can include myopathy, seizures, and hearing loss, but the common clinical feature appears to be sensory ataxia [7].

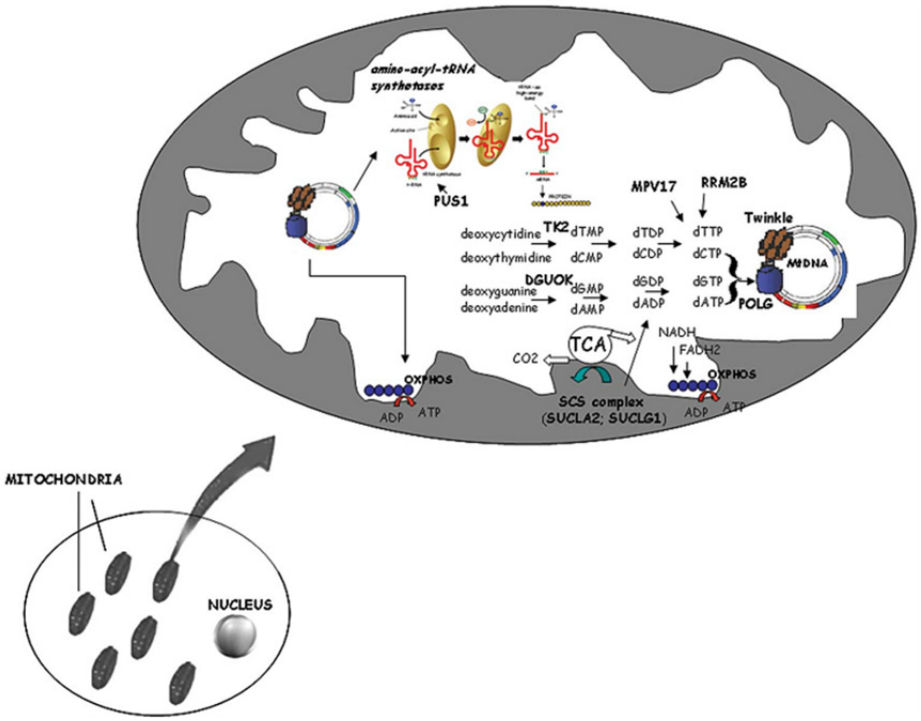

Figure 1. Schematic overview of the mitochondrion and the mitochondrial disease genes involved in intergenomic communication disorders. Zooming in on the mitochondrion allows identification of genes (namely, *POLG* and *C10orf2- Twinkle*) thought to be involved in replication of mitochondrial DNA (mtDNA); those assumed to affect the metabolism of the mitochondrial deoxynucleotide (dNTP) pool (via progressive phosphorylations of deoxythymidine, deoxycytidine, deoxytadenine, and deoxiguanosine); and those belonging to the tricarboxylic acid cycle and affecting the respiratory chain complexes (OXPHOS). Moreover, the supposed role of genes involved in the complex machinery of mitochondrial protein synthesis (including the aminoacyl-tRNA synthetases) is illustrated. This figure was kindly provided by Prof. Filippo M. Santorelli.

iii. **MNGIE** (**M**itochondrial **N**euro**G**astroIntestinal **E**ncephalomyopathy), an autosomal recessive disorder clinically characterized by onset between the second and fifth decades of life, PEO, gastrointestinal dysmotility (often pseudo-obstruction), cachexia, diffuse leukoencephalopathy, peripheral neuropathy and early death. Mitochondrial DNA abnormalities can include depletion, multiple deletions, and point mutations [8].
iv. **SCAE** (**S**pino**C**erebellar **A**taxia – **E**pilepsy syndrome) disorder similar to SANDO but with a higher frequency of migraine headaches and seizures [9].

2.2. mtDNA depletion syndromes

Quantitative alterations are characterized by depletion of mtDNA. Mitochondrial DNA depletion syndrome (MDS) comprises a heterogeneous group of autosomal recessive

disorders, all having the same molecular end result, low mtDNA amount in specific tissues. MDS are a group of rare and devastating diseases that manifest typically, although not exclusively, soon after birth, determining early death usually in infancy or early childhood. MDS differs from other respiratory chain disorders, as most often it may manifest solely in a specific organ (most commonly muscle or liver) [10]. However, it may occur that multiple organs, including heart, brain, and kidney are affected [11]. An extensive review on MDS was recently published [12].

Three major clinical categories can be recognized however, the clinical phenotypes are heterogeneous, overlapping and ever expanding [10,13]:

i. **Hepatocerebral MDS** is most probably the most common variant of MDS; Onset of symptoms is between birth and 6 months; death usually occurs within one year of age. The most common symptoms and signs include persistent vomiting, failure to thrive, hypotonia and hypoglycemia associated with progressive neurological symptoms. Histological changes on liver biopsy include fatty degeneration, bile duct proliferation, fibrosis, and collapse of lobular architecture. Reduced COX histochemistry and combined deficiency of mtDNA encoded MRC complexes were found in the liver of a few patients.

   A peculiar form of hepatocerebral MDS is Alpers-Huttenlocher syndrome, an early onset, fatal disease, characterized by hepatic failure, intractable seizures, evolving into *epilepsia partialis continua*, and global neurological deterioration. The liver dysfunction is usually progressive as well, evolving from microvesicular staetosis with bile duct proliferation into cirrhosis and chronic liver failure.

ii. **Myopathic MDS** typically onset of symptoms usually occur in the first year of life with feeding difficulty, failure to thrive, hypotonia, muscle weakness and occasionally PEO. Death is usually due to pulmonary insufficiency and infections, but some patients survive into their teens [14,15]. Muscle biopsy may show proliferation of mitochondria, which can increase with age, and patchy or diffuse COX deficiency. Biochemical defects of all mtDNA-related respiratory chain complexes are always present in muscle mitochondria. Serum CK levels may be variably elevated [4].

iii. **Encephalomyopathic MDS** is characterized by infantile onset of hypotonia with severe psychomotor retardation, high lactate in blood, progressive neurologic deterioration, a hyperkinetic-dystonic movement disorder, external ophthalmoplegia, deafness, generalized seizures and variable renal tubular dysfunction. Brain MRI was suggestive of Leigh syndrome [11].

3. Molecular etiologies of disorders affecting mtDNA integrity

In the next sections we will mention the genes identified so far, to be responsible with these disorders. Table 1 summarizes the mutations described and the associated phenotypes.

Gene	Phenotype	Mutation Type							
		M/N	Sp	Sd	Si	Sid	Gd	Gi	Gr
POLG	PEO	61		1	2				
	Alpers	37	5	2	6	2	3		
	MDS	6		1					
	Encephalopathy	3							
	Mitochondrial spinocerebellar ataxia and epilepsy	1							
	SANDO	1			1				
	POLG deficiency	29	1	2	2				
	OXPHOS deficiency	2							
	Ataxia	6	1						
	Epilepsy	2		1					
	Complex I deficiency	1							
	Others	18	1						
POLG2	PEO	1							
	Mitochondrial disease	7		1				1	
SLC25A4	PEO	5							
	Mitochondrial myopathy & hypertrophic cardiomyopathy	1	1	1	1				
SLC25A3	Muscular hypotonia & hypertrophic cardiomyopathy	1	1						
C10orf2	PEO	34				1		1	
	MDS	1							
	Spirocerebellar ataxia, infantile onset	2							
	Cholestatic liver disease	1							
	Encephalopathy	1							
	Ocular myopathy	1							
	Complex I deficiency	1							
	Dementia	1							
	Reduced expression	1							
Tymp	MNGIE	47	11	11	6	1			
TK2	MDS	20	1	2	9	1	1		1
	Epileptic encephalomyopathy				1				
	MDS with hearing loss				1				
	PEO	1							
DGUOK	MDS	28	6	9	4		1		
RRM2B	MDS	12	2	1					
	PEO	7		1	1				
	MNGIE	2							
	KSS	1							

Gene	Phenotype	Mutation Type							
		M/N	Sp	Sd	Si	Sid	Gd	Gi	Gr
MPV17	Altered p53 binding, association with MDS – hepatocerebral	7	1 1	1		1	2		
	Liver failure in infancy			2					
	Lethal hepatopathy & leukodystrophy		1						
SUCLA2	MDS – encephalomyopatic & methylmalonic ac.	4	1			1			1
SUCLG1	Succinyl-coenzyme A synthetase deficiency	4	1	1					
	Lactic acidosis with mitochondrial DNA depletion	2		1					
	Neonatal lactic acidosis with methylmalonic aciduria	2							
	Lactic acidosis, fatal infantile		1						
	Mitochondrial hepatoencephalomyopathy	1							
PUS1	Mitochondrial myopathy and sideroblastic anaemia	2							
	Sideroblastic anaemia	1							
TRMU	Infantile liver failure, increased risk	6	1	1					
	Combined OXPHOS deficiency				2				
	Phenotype modifier	1							
	Respiratory chain deficiency, reversible infantile		1						
LRPPRC	Cytochrome c oxidase deficiency	1				1			
TACO1	Cytochrome c oxidase deficiency				1				
TUFM	Combined OXPHOS deficiency	1							
TSFM	Combined OXPHOS deficiency	2							
GFM1	Combined OXPHOS deficiency	8		2					
MRPS16	MRC disorder	1							
RARS2	Pontocerebellar hypoplasia	3	1	1					
DARS2	Leukoencephalopathy, brain & spine involvement, lactate elevation	15	11	1		2	1		
	Episodic ataxia, exercise-induced	1							
YARS2	MLASA syndrome	1							

Table 1. Mutations types described in genes involved in mtDNA integrity and mitochondrial translation and associated clinical phenotype (M/N- missense/nonsense; Sp- splicing; Sd- small deletions; Si- small insertions; Sid- small inddels; Gd- gross deletions; Gi- gross insertions; Gr- gross rearrangements) - source HGMD Professional database www.hgmd.cf.ac.uk/.

3.1. Genes involved in mitochondrial replisome

3.1.1. POLG

Human mitochondria contain a single DNA polymerase, Polymerase gamma (POLγ), nuclear encoded and solely responsible for mtDNA replication and repair in mitochondria. POLγ is composed of a catalytic subunit, POLγA, which possesses both polymerase and proofreading exonuclease activities and an accessory subunit, POLγB, which increases enzyme processivity [16]. The POLγ holoenzyme functions in conjunction with the mitochondrial DNA helicase and the mitochondrial single-stranded DNA- binding protein to form the minimal replication apparatus [17]. It was generally accepted that mutations within the mtDNA were the major cause of mitochondrial diseases; however this view is changing as several of these have been linked to ineffective mtDNA replication by POLγ.

Mutations affecting the catalytic subunit POLγA, encoded by the nuclear gene *POLG* are a major cause of mitochondrial disease, being highly heterogeneous – PEO, Parkinsonism, AHS, MNGIE, SANDO and SCAE- and usually is associated with multiple mtDNA deletions [18]. POLG mutations have been shown to be associated with all types of inheritance. The unique features of mitochondrial physiology are in part responsible for this variability but *POLG* structure and function add to the riddle of how one gene product can demonstrate autosomal recessive and autosomal dominant transmission. POLγA is a key player in mtDNA maintenance that is absolutely necessary for mtDNA replication from an early stage in embryogenesis [19]

In adPEO due to *POLG* mutations (most frequent), prominent features are severe dysphagia and dysphonia, and, occasionally, a movement disorder including Parkinsonism, cerebellar dysfunction, and chorea. Recessive mutations of *POLG* are responsible for sporadic and arPEO, as well as the syndromes referred above. Mutations in this gene can be also associated to the hepatocerebral form of MDS, namely AHS [18].

The *POLG* gene is located at chromosome 15, comprises 23 exons spanning 18.55 Kb. The gene was identified in 1996 [20] but only in 2001 the first pathogenic mutation was described. Since then more than 150 mutations have been reported and POLG gene is considered a hot-spot for mutations in mitochondrial diseases [21].

3.1.2. POLG2

MtDNA is replicated by DNA polymerase gamma, which is composed of a 140-kD catalytic subunit (encoded by *POLG*) and a 55-kD accessory subunit (*POLG2*). The accessory subunit increases enzyme processivity therefore it is not surprising that failure in this processivity leads to the accumulation of mtDNA deletions.

The *POLG2* gene is located at chromosome 17, comprises 8 exons spanning 19.28 Kb. In 2006 the first pathogenic mutation was described as being a cause of adPEO [22]. Since then, 10 mutations in *POLG2* have been reported.

3.1.3. C10orf2 (Twinkle)

The mitochondrial helicase/primase encoded by *C10orf2* gene is also responsible for the adPEO [23]. Mutations in *C10orf2* may be of variable severity, being associated with clinical presentations ranging from late-onset ''pure'' PEO, to PEO complicated by proximal limb and facial muscle weakness, dysphagia and dysphonia, mild ataxia, and peripheral neuropathy. Recessive *C10orf2* mutations were also described in patients with hepatocerebral form of MDS [24].

The *C10orf2* gene is located at chromosome 10; it comprises 5 exons spanning 6.38 kb. The first pathogenic mutation was reported in 2001 [23] to be associated with PEO and since then 45 pathogenic mutations have been reported.

3.2. Genes involved in the synthesis and supply of nucleotide pools

3.2.1. SLC25A4

This gene, coding for the muscle-heart-specific mitochondrial adenine nucleotide translocator (ANT) is a member of the mitochondrial carrier subfamily of solute carrier protein genes [25]. ANT is the most abundant mitochondrial protein and in its functional state, it is a homodimer of 30-kD subunits embedded asymmetrically in the inner mitochondrial membrane. The dimer forms a gated pore through which ADP is moved from the matrix into the cytoplasm. There are three recognized isoforms of this protein.

Mutations in this gene have been shown to be responsible for the adPEO and have been also associated with a relatively mild, slow progressive myopathy, with little or no extramuscular symptoms.

The *SLC25A4* gene was identified in 2000 [25], it is located at chromosome 4, comprises 4 exons spanning 4.04 Kb. The first pathogenic mutations were described in 2000 and since then only seven mutations have been reported (most of them associated with PEO).

3.2.2. SLC25A3

The *SLC25A3* gene codes for a mitochondrial phosphate carrier. A defect in this mitochondrial phosphate carrier has been described in two children with hypertrophic cardiomyopathy, muscular hypotonia, severe growth retardation and death in the first year of life [26].

The gene is located at chromosome 12, comprises 7 exons spanning 8.37 Kb. The first pathogenic mutations were described in 2007 [26] and since then only one more mutation has been reported.

3.2.3. Tymp (ECGF1)

The *Tymp* gene, responsible for MNGIE (Mitochondrial NeuroGastroIntestinal Encephalomyopathy), encodes the enzyme thymidine phosphorylase (TP), which is

involved in pyrimidines catabolism. Defects of TP result in systemic accumulation of thymidine and deoxyuridine, which leads to deoxynucleotide pool imbalance and mtDNA instability, resulting in the presence of multiple deletions and partial depletion of muscle mtDNA [27].

The *Tymp* gene is located at chromosome 22 it comprises 10 exons spanning 4.3 kb. The first pathogenic mutations were described in 1999 [27] and since then 65 mutations have been described as being associated with MNGIE.

3.2.4. TK2

Thymidine kinase (TK2) is an intramitochondrial pyrimidine nucleoside kinase that phosphorylates deoxynucleotides (dNTPs), such as: deoxythymidine, deoxycytidine, and deoxyuridine, thereby participating in the salvage pathway of deoxynucleotide synthesis in the mitochondria [28]. Mitochondrial dNTPs pools arise either through active transport of cytosolic dNTP or through salvage pathways. Both pathways are essential for the replication of mtDNA, since the mitochondrion is unable to synthesize dNTPs *de novo*. Mutations in the *TK2* gene on chromosome 16q22 affect primarily muscle tissue, with little or no effect on the liver, brain, heart, or skin. The typical manifestation of *TK2* mutations is a severe, rapidly progressing myopathy of infantile or childhood onset. The disease course is rapidly progressive, leading to respiratory failure and death in months or years, but milder phenotypes with slower progression and longer survival have been reported [10]. Since the first mutation was described in 2001 [29], approximately 25 different pathogenic mutations in *TK2* have been published so far, either as recessive homozygous or compound heterozygous mutations, and phenotypes may be explained by variable degrees of residual activity of the mutant enzymes.

3.2.5. DGUOK

Deoxyguanosine kinase is a 2-deoxyribonucleoside enzyme that catalyzes the first step of the mitochondrial deoxypurine salvage pathway, the phosphorylation of purine deoxyribonucleosides into the corresponding nucleotides deoxyguanosine and deoxyadenosine necessary for the maintenance of mitochondrial dNTPs pools [11,30]. The typical phenotype of mutations in the *DGUOK* gene, on chromosome 2p13, is characterized by neonatal onset of progressive liver disease and feeding difficulties, usually with neurological dysfunction (hypotonia, nystagmus, and psychomotor retardation), by the age of 3 months. Peripheral neuropathy and renal tubulopathy have occasionally been reported [31]. Depletion of mtDNA has been documented only in the liver and results in combined respiratory chain deficiencies in the liver, whereas the amount of mtDNA is usually normal in muscle and fibroblasts. Histological analyses of the liver biopsy show variable findings, typically microvacuolar steatosis, cholestasis, fibrosis, and cirrhosis. In most cases, there is a rapidly progressive liver disease and neurological deterioration, with death occurring by the age of 12 months or shortly thereafter [32]. The first pathogenic mutations was reported in

2001 [33], since then more than 80 affected patients from approximately 50 families have been reported, and over 40 different *DGUOK* mutations have been identified [10]. The infantile hepatocerebral form of MDS is the almost invariable clinical presentation. Genotype-phenotype correlation studies show that patients who harbor null mutations usually have early onset liver failure and significant neurological disease, including hypotonia, nystagmus, and psychomotor retardation, and death before two years of age. Patients carrying missense mutations usually have isolated liver disease, a better prognosis, and longer survival.

3.2.6. RRM2B

The *RRM2B* gene on chromosome 8q23 encodes the small subunit of p53-inducible ribonucleotide reductase, a heterotetrameric enzyme responsible for *de novo* conversion of ribonucleoside diphosphates into the corresponding deoxyribonucleoside diphosphates that are crucial for DNA synthesis [34]. The enzyme is the main regulator of the nucleotide pools in the cytoplasm, and its small subunit is expressed in postmitotic cells, where it probably has a key function in maintaining the mitochondrial dNTPs pools for mtDNA synthesis. Mutations in *RRM2B* usually result in hypotonia, lactic acidosis, failure to thrive, and tubulopathy in the first months of life. The disease has a rapid progression and leads to death in a few months. The associated complex phenotype suggests that the consequences of a defective mitochondrial dNTPs pools can vary dramatically depending on the residual amount of the functional enzyme. Recently, it has been shown that inactivating mutations in *RRM2B* also cause severe neonatal or infantile forms of mtDNA depletion, with profound reduction of mtDNA copy numbers in skeletal muscle [34]. The first pathogenic mutation was reported in 2007 [34] and since then 26 mutations have been described.

3.2.7. MPV17

The *MPV17* gene is located on chromosome 2p23-p21 and encodes a mitochondrial inner membrane protein of unknown function recently recognized as responsible for mtDNA depletion. The clinical presentation is that of severe liver failure, hypoglycemia, growth retardation, neurological symptoms, and multiple brain lesions during the first year of life [35]. Marked mtDNA depletion in the liver is the molecular hallmark associated with multiple defects of respiratory chain complexes. Normal or mildly reduced levels of both mtDNA content and respiratory chain enzyme activities were also found in muscle [36]. Histological analyses of the liver have revealed swollen granular hepatocytes, microvesicular steatosis, and focal pericellular and periportal fibrosis. Since the first mutation was described in 2006 [37], about 15 different mutations have been reported in infantile-onset hepatocerebral syndrome and in Navajo neurohepatopathy, which is an autosomal recessive multisystem disorder found in the Navajo of the southwestern United States [30]. Three main subtypes are to be considered: infantile-onset (before 6 months) and

childhood-onset (before 5 years) forms with hypoglycemic episodes and severe progressive liver dysfunction requiring liver transplant, and a 'classic'' form with moderate hepatopathy and progressive sensorimotor axonal neuropathy. The three forms are also associated with variable degrees of demyelination in both the central and the peripheral nervous system.

3.2.8. SUCLA2 and SUCLG1

Succinyl CoA synthase is a mitochondrial matrix enzyme that catalyzes the reversible synthesis of succinate and ATP or GTP from succinyl-CoA and ADP in the tricarboxylic acid cycle. This enzyme is made up of two subunits, a and b, encoded by *SUCLG1* on chromosome 2p11 and *SUCLA2* on 13q12, respectively. Mutations in *SUCLA2* and *SUCLG1* cause an encephalomyopathic form of infantile mtDNA depletion syndrome, but *SUCLG1* can also cause a very severe disorder with antenatal dysmorphisms, neonatal metabolic crisis, and early death, probably depending on the lower residual amount of the protein [38,39]. A useful diagnostic clue in Succinyl CoA synthase disorders of succinyl CoA synthase is a ''mildly'' elevated urinary methylmalonic acid, which is detected in all patients, and presence of tricarboxylic acid cycle intermediates (methylcitrate, lactate, carnitine esters, 3-hydroxyisovalericacid) in most cases. Some patients die as infants (sudden infant death syndrome), but some of them have a longer survival. The clinical features of patients with mutations in these genes include early childhood hypotonia, developmental delay, and almost invariably, progressive dystonia and sensorineural deafness. *SUCLA2* and *SUCLG1* mutations seem to disrupt an association between succinyl CoA synthase and mitochondrial nucleoside diphosphate kinase, resulting in an unbalanced mitochondrial dNTP pool and eventually, mtDNA depletion in muscle. The first pathogenic mutations were reported in 2005 [40] and 2007 [41] in *SUCLA2* and *SUCLG1*, respectively and since then few mutations have been described.

3.3. Genes involved in mitochondrial translation

Mendelian diseases characterized by defective mitochondrial protein synthesis and combined respiratory chain defects have also been described in infants and are associated with mutations in nuclear genes that encode components of the translational machinery, such as those encoding elongation factors, aminoacyl-tRNA synthetases, or even mtDNA encoded tRNA [12]. Mitochondria contain a separate protein-synthesis machinery to produce the polypeptides encoded in mtDNA, and many mtDNA disease mutations affect this machinery. This group of disorders is highly heterogeneous and usually shares a combined disorder of respiratory chain complexes.

3.3.1. Genes involved in mitochondrial translation factors

3.1.1.1. PUS1

The Pseudouridine synthase 1 (*PUS1*) gene on chromosome 12q24 encodes an enzyme that converts uridine into pseudouridine at several cytoplasmic and mitochondrial tRNA

positions and thereby improves translation efficiency in the cytosol as well as the mitochondrion. Thus, *PUS1* is not part of the translation machinery, but it is required for protein synthesis because of its function in posttranscriptional modification of tRNA. Mutations in *PUS1* are responsible for the rare myopathy, lactic acidosis, sideroblastic anemia syndrome and sometimes include mental retardation. The first pathogenic mutation was reported in 2004 [42] and since then few mutations have been described.

3.1.1.2. TRMU

The *TRMU* gene on chromosome 22q13 encodes an evolutionarily conserved protein involved in mitochondrial tRNA modification and is important for mitochondrial translation. Defects in tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase (TRMU), a mitochondria specific enzyme that is required for the 2-thiolation on the wobble position of the tRNA anticodon, result in reduced steady-state levels of 3 tRNA (tRNALys, tRNAGln, and tRNAGlu) and consequently, impaired mitochondrial protein synthesis [43,44]. Recently, mutations in *TRMU* were detected in patients with acute liver failure in infancy [44].

3.1.1.3. LRPPRC

The *LRPPRC* gene is located on chromosome 2p21. Leucine-rich PPR-motif containing protein has been suggested to function together with heterogeneous nuclear ribonucleoprotein K and RNA polymerase in coupling the mitochondrial transcription and translation machineries [45]. Mutations in *LRPPRC* lead to the French-Canadian subtype of Leigh syndrome, associated with a profound deficiency of complex IV of the OXPHOS system [46]. Patients exhibit neonatal or infantile onset hypotonia and psychomotor delay, and bilateral hyperluciencies of basal ganglia, like other more common forms of Leigh syndrome. The first pathogenic mutation was reported in 2003 [46] and since then one more mutation has been described.

3.1.1.4. TACO1

TACO1 represents the first specific mammalian mitochondrial translational activator, opening the possibility to a new class of proteins controlling efficiency of mitochondrial translation. Mutations in *TACO1*, located on chromosome 17q.6, are responsible for a relatively late-onset Leigh syndrome (onset range 4-13 years) characterized by short stature, mental retardation with autistic-like features, and a slowly progressive array of motor symptoms related mainly to basal ganglia involvement [47,48]. Only one mutation was described to date [47].

3.1.1.5. TUFM, TSFM and GFM1

Another important player during mitochondrial protein biosynthesis is the group of elongation factors. The mitochondrial EF-Tu forms a ternary complex with tRNA and GTP and promotes the binding of tRNA to the ribosome. A few patients have been described as

having mutations in genes encoding components of the mitochondrial translation elongation machinery, including elongation factor EF-Tu (TUFM), EF-Ts (TSFM) and EFG1 (GFM1). These patients have severe disease, presenting neonatal lactic acidosis and neurological impairment resembling Leigh syndrome, leading to early fatality. The first pathogenic mutations in these genes were reported recently [49,50,51] and since then few mutations have been described.

3.1.1.6. MRPS16 and MRPS22

Of all 81 human mitochondrial ribosomal proteins (MRPs), mutations have been found in only two, MRPS16 and MRPS22 [52,53]. Both defects resulted in a marked decrease in the 12S rRNA transcript level, probably caused by impaired assembly of the mitoribosomal small subunit, generating unincorporated and unstable 12S rRNA. Indeed, lack of MRPs results in the failure to assemble parts of small subunits of the mitoribosome, and subsequent degradation of its components [54]. Clinical manifestations include agenesis of the *corpus callosum*, dysmorphism, hypertrophic cardiomyopathy, and fatal neonatal lactic acidosis. The first pathogenic mutations were reported in 2004 [52] and in 2007 [53], and since then few mutations have been described.

3.3.2. Genes involved in mitochondrial aminoacyl tRNA synthetases

3.3.2.1. RARS2, DARS2, and YARS2

To guarantee fidelity in translation, it is important to attach the right amino acid to the tRNA and to ensure that the tRNA recognizes, through its anticodon, the correct codon in the ribosomal A-site. Incorporation of an incorrect amino acid into the nascent polypeptide could cause misfolding and production of defective or dominant interfering proteins. Amino acids are attached to tRNA by amino-acyl-tRNA synthetases, each of which is specific for a single amino acid. However, as there can be several codons and several different tRNA for a single amino acid, an amino-acyl-tRNA synthetase can ''charge'' several different tRNA. If this function is defective, certain codons will become ambiguous, resulting in the synthesis of misfolded proteins, which could aggregate to form inclusions and induce further protein misfolding. Mutations in the *RARS2* and *DARS2* were recently described [55,56,57] and are associated with severe encephalopathy with pontocerebellar hypoplasia and leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation, respectively, with most patients showing onset between 2-15 years of age [56]. Very recently, mutations in the gene encoding the mitochondrial *YARS2* have been associated with a clinical condition characterized by myopathy, lactic acidosis, and sideroblastic anemia [54].

4. Diagnostic approaches for intergenomic communication disorders

Suspicion of intergenomic communication disorders arising from clinical presentation may range from well defined syndromes to unspecific multisystemic phenotype, where neurological involvement is usually present.

Establishing a specific diagnosis in a patient with suspected mendelian disease is a challenging task that requires the integration of clinical assessments, family history, biochemical testing and histopathological examination. It is important to obtain the appropriate biochemical and/or clinical information before starting any molecular investigations so that molecular diagnosis can be successfully.

Biochemical determination of mitochondrial respiratory chain complexes is important for delineating the molecular approach in particular in patients without a specific neurological syndrome. As mtDNA encodes for subunits of respiratory chain complexes I, III, IV and the ATP-synthase, mtDNA depletion causes a combined respiratory chain deficiency of all complexes, except complex II. Biochemical analysis of the muscle respiratory chain enzyme activities may, however, be normal, if skeletal muscle is not among the affected tissues, e.g., in MDS of the brain or liver. Southern analysis or quantitative real-time polymerase chain reaction are two methods that simultaneously detects mtDNA deletion(s) and quantify total mtDNA content. In both approaches, mtDNA amount is compared to a specific nuclear reference gene. A prerequisite for correct interpretation of mtDNA amount is to consider the dynamic nature of mtDNA amount in different ages and tissues, and therefore to establish carefully age-matched control materials [58]. A reduction in mtDNA copy number to 60-65% of age-matched controls has been established for an empirical cut-off level for MDS diagnosis, but especially in children, the reduction may be severe (80-90%). Biochemical data, such as lactate, pyruvate, alanine, organic acid profiles as well as neuroimaging findings are also important clues for the diagnosis of these disorders. Some diagnostic clues exist for specific gene defects: serum creatine kinase (CK) is elevated in *TK2* defects, serum thymidine in *TYMP* defects and urine methylmalonic acid and methylcitrate in *SUCLA2* and *SUCLG1* defects [10].

The *POLG* gene seems to be the most frequently mutated nuclear gene in cases of mitochondrial disease therefore in cases of normal mtDNA testing and clinical signs such as nonspecific hypotonia, developmental delay, epilepsy and progressive liver disease *POLG* gene investigation should be considered. Valproate-induced liver toxicity in *POLG* and *C10orf2*-MDS emphasizes the importance of diagnosing these patients, who usually suffer from severe treatment-resistant epilepsy [59]. We suggest *POLG* analysis before valproate treatment for such children and adolescents, whose first epileptic attack develops to a status epilepticus of unknown cause.

Based on our practice, we present a testing algorithm for establishing an accurate diagnosis for these diseases (Figure 2).

5. Therapeutic considerations

The management of mitochondrial disease is largely supportive as no curative therapy is available. Palliative/supportive treatment with vitamins, cofactors and respiratory substrates have been used, but with poor efficacy. In the last years several approaches have been tried and the enhancement of mitochondrial biogenesis has emerged as an exciting therapeutic possibility. The enhancement of mitochondrial biogenesis might restore mitochondrial function in a variety of other contexts.

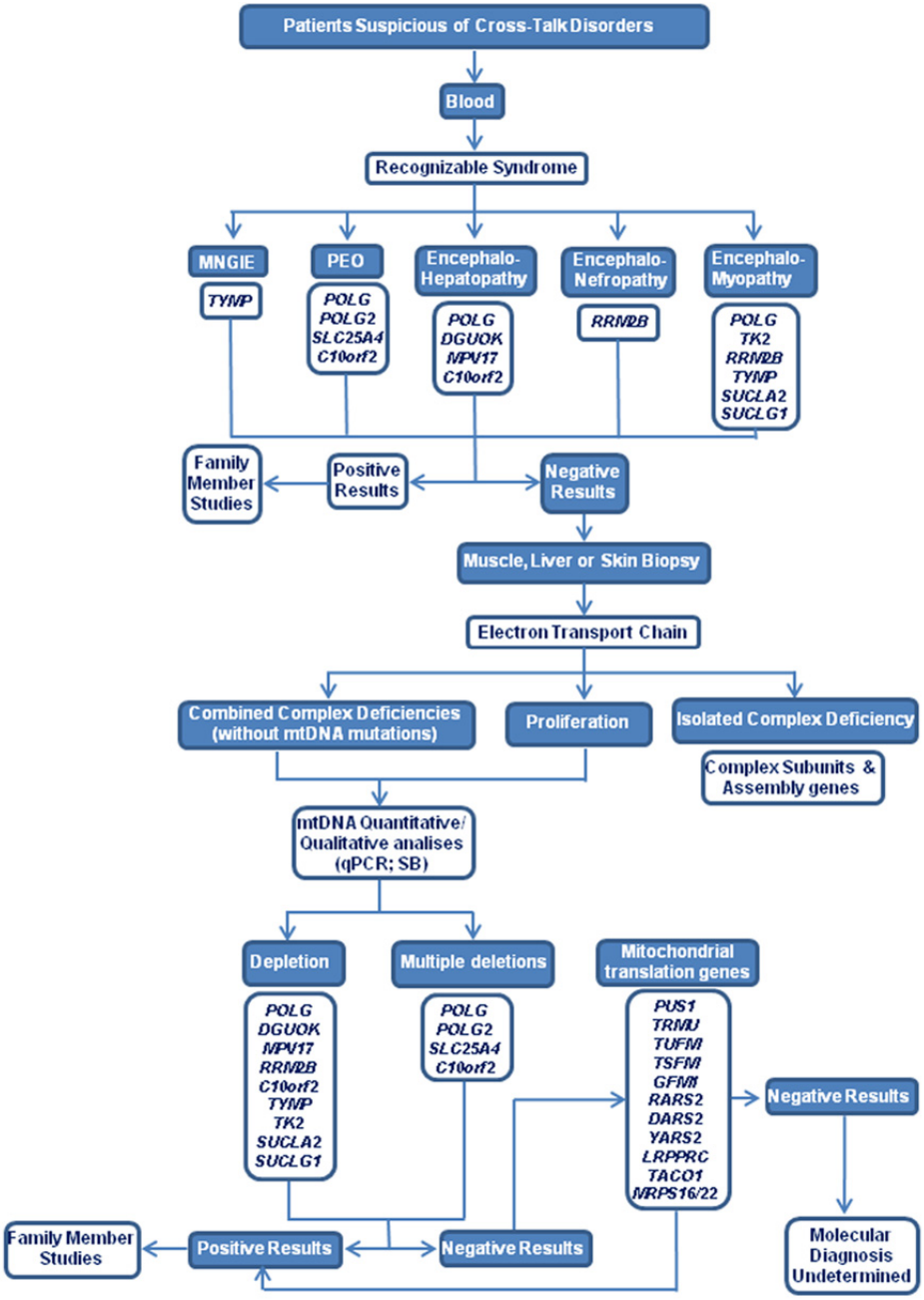

Figure 2. Diagnostic algorithm for intergenomic communication disorders, based on clinical and biochemical information.

What has been noticed is that for every case there is a different strategy. For example liver transplantation may be beneficial to patients with hepatopathy caused by *DGUOK* mutations if no neurological symptoms have developed. However, significant hypotonia, psychomotor retardation or nystagmus should be contraindications for the liver transplantation [60]. In patients with *MPV17*, liver transplantation has increased quality and years to life for some patients [61,62], but the patients have developed neurological symptoms. Some children with *POLG* mutations have received a liver transplant after valproate-induced liver failure, and although it has rescued their liver function, neurological outcome has been unfavorable [63,64].

In patients with *MPV17* mutations, a controlled diet avoiding hypoglycemias were suggested to slow down the progression of liver impairment and be useful in supportive care [65]. Some improvement of liver functions in a patient with *MPV17* mutations was gained by treating them with succinate or coenzyme-Q10 together with a lipid-rich diet [66]. Further studies with larger patient materials and longer follow-up time are needed to confirm, if these dietary interventions were beneficial, and could be recommended. In MNGIE, correlation between plasma thymidine levels and the severity of the phenotype has been observed [67]. Therefore, attempts to reduce the circulating nucleotide levels could result in disease improvement. Enzyme replacement therapy has been applied for MNGIE: infusion of platelets from healthy donors to patients with MNGIE reduced their circulating thymidine and deoxyuracile levels, and partially restored TP activity. The limitation of this therapy was the short half-life of platelets [68]. Allogenic stem cell transfusions have been given to two patients with MNGIE [69]. Although more experience is needed to illustrate the clinical benefit of that treatment, it opens up a possibility of treatment for disorders of the nucleoside metabolism. In MNGIE, also continuous ambulatory peritoneal dialysis has been used to reduce the thymidine levels, and this resulted in improvement of the symptoms during 3-year follow-up time [70]. Good animal models will enable testing these hypotheses *in vivo*.

6. Conclusive remarks

The diagnostic process in nuclear disorders of oxidative metabolism is not too different from that employed for other diseases and includes patient and family history, physical and neurologic examination, routine and special laboratory tests, muscle biopsy for morphology and biochemistry, and molecular genetics screening [71]. A mitochondrial disease manifesting at or soon after birth is more likely to be associated with nDNA than with mtDNA mutations, but until very recently, our profound ignorance regarding the mechanisms underlying mitochondrial gene transcription and translation and the complex interaction between the ''2 genomes'' has limited our diagnostic power. Mitochondrial DNA deletion and depletion syndromes, and disturbances in the mitochondrial translation machinery have become an increasingly important cause of a wide spectrum of infantile and childhood-onset multisystem disorders. Depletion syndromes could result from any imbalance of the mitochondrial dNTPs pools available for mtDNA replication, as well as abnormalities in either the mitochondrial helicase or DNA polymerase. Consistent with the

different phenotypes, mtDNA depletion may affect specific tissue (most commonly, brain and muscle or liver) or multiple organs, including the heart and the kidney. Predictably, affected tissues show paucity of mtDNA-encoded translation products and multiple respiratory chain defects. More than 75% of these patients had onset during the first year of life, and the disease was rapidly fatal in most cases [3,72,]. Moreover, though the components of the complicated mitochondrial protein-synthesis machinery are exclusively nuclear encoded, the majority of mutation affects correct translation of mtDNA-encoded subunits of the OXPHOS system and accounts for a still undetermined number of genetic defects. Indeed, there is still limited information on the many mitoribosomal proteins; the several tRNA maturation enzymes; the aminoacyl-tRNA synthetases; the translation initiation, elongation, and termination factors; and the predictably larger number of unidentified factors needed for ribosome assembly [43,73].

The increasing number of nuclear governed mitochondrial diseases and its associated genes continues to increase the diversity of the genetic and clinical phenotypic heterogeneity of this group of disorders. Identifying the causative genes is not only important for adequate genetic counseling and prenatal diagnosis but also to have a better understanding of the disease pathophysiology leading to better therapy options. The increasing number of genes involved is a driving force for the development of high throughput strategies. The recent advances on sequencing technology will facilitate the molecular investigations of genes associated with mtDNA disorders in general. Reports concerning the use of next generation sequencing for the diagnosis of mitochondrial disorders are emerging [74,75,76]. In a recent report the use of target NGS for mitochondrial disorders proved its efficiency in clinical diagnosis as for 55% of the studied patients a clear molecular etiology was found. As more studies are reported the importance of applying this technology will be highlighted.

The problems faced by patients with mitochondrial respiratory chain disease are particularly severe. Diagnosis is difficult, treatment is largely ineffective, genetic counseling and prenatal diagnoses are uncertain or unavailable and the prognosis is unpredictable. Because diagnosis is imperfect and laborious, many patients undergo a whole battery of unnecessary investigations during the diagnostic process. Accurate focused diagnosis will save time, money and distress. Only by understanding the molecular genetic basis of these disorders, whether nuclear or mitochondrial, will any progress be made. Furthermore this will help patients, but will also lead to fundamental advances in our understanding of mitochondrial biology. Identification of new disease-causing gene(s) will hopefully provide insights towards novel therapeutic strategies.

Chapter highlights

- The chapter focus on diseases of intergenomic communication disorders mainly the ones involved in mtDNA integrity and mitochondrial protein synthesis
- Disorders affecting mtDNA stability lead to multiple deletions or depletion of mtDNA
- This group of disorders can affect a variety of organ with variable ages of onset
- *POLG* is frequently mutated being a hotspot for mitochondrial disease

- Diagnosis is difficult and laborious due to the increasing number of genes involved
- Therapy in mainly palliative however novel strategies are emerging
- Due to the increasing number of genes involved novel diagnostic strategies are emerging to optimize the diagnosis offered to these families

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Chapter 14

Myofibrillar Myopathies and the Z-Disk Associated Proteins

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Additional information is available at the end of the chapter

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1. Introduction

Myofibrillar myopathies (MFMs) are typically autosomal dominant myopathies with late onset progressive muscle weakness and symptoms initially evident in the distal muscle groups. However, there is a significant variability in the presentation of these diseases, with the age of onset ranging from infantile to late seventies; the involvement of the heart, respiratory muscles, distal or proximal muscle groups; and severity covering the full spectrum from mild muscle weakness to premature lethality. Several myopathies were identified with symptoms within this broad spectrum and the recognition of a common pathology allowed the grouping of these diseases under a single term, MFM [1]. Problems in the classification of these disorders still exist, partially due to the wide spectrum of clinical presentation and the lack of detailed analysis of biopsy samples to identify the defining features of MFM.

The defining features of MFM, identified using histological stains and electron microscopy, are the dissolution of muscle fibres and the formation of protein aggregates. Common pathological features of MFM include presence of amorphous, granular, filamentous or hyaline deposits, interstitial fibrosis, fatty infiltration, centrally located nuclei indicative of regeneration, necrosis and muscle degeneration. Displaced membranous organelles are also evident, either in the cytoplasm or within autophagic vacuoles. Affected areas of the cells are frequently devoid of oxidative enzymatic activity and mitochondria can be abnormally shaped and positioned [2-5]. Characterization of the protein aggregates using immunohistochemistry reveals the presence of a wide range of sarcomeric, extracellular, and ubiquitously expressed proteins including Myotilin, Desmin, αB-Crystallin, Filamin C, BAG3, ZASP, Actin, Titin, Myosin, Xin, Dystrophin, sarcoglycans, Plectin, Delsolin, Ubiquitin, Neural cell adhesion modulator, Gelsolin, Syncoilin, Synemin, TAR DNA-binding protein 43, Heat-shock protein 27, and DNAJB2 [6]. Interestingly, α-Actinin, which

is the primary Z-disk crosslinker and is associated with many of the Z-disk proteins mutated in MFM, is not detected in these protein aggregates [6,7].

The Z-disk provides an important structural linkage in the transmission of tension and contractile forces along the muscle fibre and has a role in sensing of muscle activity and signal transduction. In line with the identification of the Z-disk as the primary site affected in these myopathies the identification of MFM causing mutations has revealed a very strong association with the Z-disk, with all of the proteins affected being localised to this structure. Mutations have been identified in the intermediate filament (IF) protein Desmin [8], the chaperone αB-Crystallin [9], the structural protein Myotilin [10], the α-Actinin binding protein ZASP [11], the actin binding protein Filamin C [12], and the co-chaperone BAG3 [13]. Based on the Mayo Clinic MFM cohort, 14% of MFMs are due to mutations in ZASP, 13% due to Myotilin mutations, 8% Desmin mutations, 5% αB-Crystallin mutations, 4% BAG3 mutations, and 4% due to mutations in Filamin C, with the genetic basis of more than 50% of MFM cases remaining unknown [14].

Whilst subtle differences in morphology and histochemical staining are found to be associated with certain MFM subtypes [3], they are not reliable in identifying the genetic cause of MFM. Ultrastructural studies on the other hand have been shown to be more informative in identifying the subtype of MFM, although repetition with large sample sizes is required to determine the reliability of ultrastructure studies is directing diagnosis [15].

Mutations in any of the identified MFM genes can also result in other forms of myopathy including dilated cardiomyopathy (DCM), restrictive cardiomyopathy (RCM), distal myopathy, spheroid body myopathy (SBM), and limb-girdle muscular dystrophy (LGMD). Whilst mutations can result in different myopathies, within the cases of MFM there is a remarkable consistency in the phenotype regardless of the gene mutated. This unifying pathological presentation suggests a common mechanism of pathology, although the functions of the MFM proteins and how their mutation results in disease are not fully understood. Determination of the mechanism by which these mutation result in disease will not only be important for the development of therapies for these conditions but will also provide insight into the role of these proteins in the muscle and the many functions of the Z-disk. We present an analysis of the literature surrounding each of these proteins and how their mutations result in disease and discuss the implications for MFM and Z-disk function.

2. Desmin and desminopathies

Desmin, named from the word 'desmos' which means 'link' is a small, 53KDa, IF protein found in skeletal, smooth [16], and cardiac [17] muscle cells. In mature skeletal muscle, Desmin along with other Desmin-binding molecules such as Plectin, links adjacent myofibrils at the Z-disk and binds them to the sarcolemma at the costameric level [18]. Desmin localisation to the intermediate filament, Z-disk, and costamere provides a cytoskeletal network that links the contractile apparatus to the cell membrane and other structural elements of the cell, which is critical for maintaining the integrity of the cell, ensuring force transmission and providing with a pathway for signalling. In order to form a fully functional IF network Desmin connects

with different cell structures from the cell membrane to the nuclear envelope. Therefore Desmin interacts with a range of different muscle, non-muscle, and nuclear proteins. At the Z-disk, it interacts with αB-Crystallin (CRYAB) [19] and Nebulin [20,21]. At the periphery of the Z-disk, costameres, nucleus, and neuromuscular junctions Desmin interacts with Vimentin, Synemin [22], Paranemin [23], Desmulsin [24], Lamin [25], Plectin [26], Nestin [27], spectrins [28], and Ankyrin [29]. Deficiency in Desmin not only results in disturbance to the structure of the sarcomere, but also results in striking changes to the cellular morphology, which may have direct implications for muscle function. Desmin knockout mice show abnormal mitochondrial localization, accompanied by an increase in number and size, a rounded shape and distorted membranes, often showing granules and even mineralised bodies [30].

Structurally, Desmin is made up of three domains; an N-terminal head domain, a highly conserved central α-helical core, and a C-terminal tail domain (Figure 1). The central α-helical core, a region responsible for Desmin assembly into IF, is made up of four consecutive helical segments, 1A, 1B, 2A and 2B, which are linked by short non helical linkers [31,32]. These helical domains are made of tandem repeats of a specific seven amino acid sequence that contains the biochemical properties that allow the proper coiling of the protein. Additionally, the 2B helical domain contains a four amino acid insertion, known as the 'stutter', critical for Desmin assembly and conserved between many IF proteins [33,34]. Of the 50 Desmin mutations reported so far that result in severe skeletal and/or cardiac muscle defects the majority affect the coiled domains, five affect the head domain and eleven affect the tail domain (Figure 1). Interestingly, no mutations in domain 2A have been reported to date and more than 50% of reported Desmin mutations are in the 2B domain [35]. Although a correlation between the domain mutated and the clinical features of the patients/carriers has been suggested (reviewed in [35]), when the clinical features are analysed in more detail the only correlation that appears to be maintained is the predominance of skeletal muscle defects in patients with mutations in the 2B domain (Table 1 and Table 2).

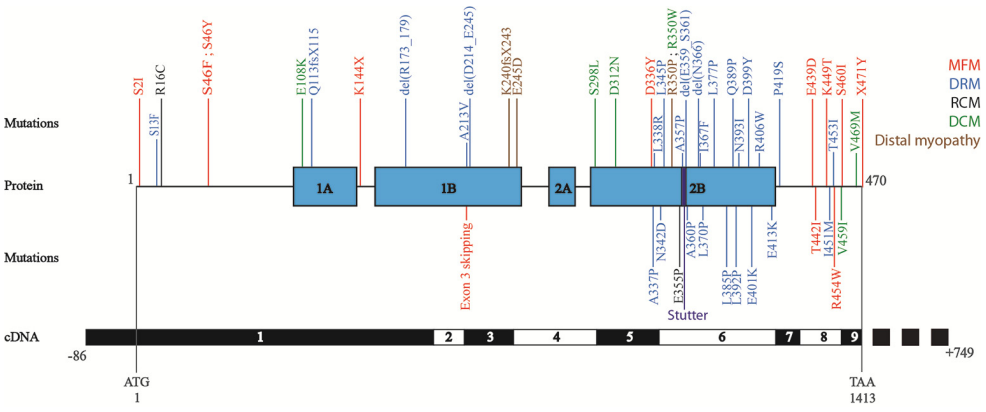


Figure 1. Schematic representation of Desmin domains and mutations.

Mutations are coloured accordingly to the disease classification. Note that 3′UTR is not drawn to scale. Mutations are coloured accordingly to the disease classification. Note that 3′UTR is not drawn to scale.

Mutation	Age of onset / Initial symptoms	Clinical and pathological features; other studies	Ref
c.5G>T **S2I**	? Skel	**Skel:** MW**. Pathology:** abnormal myofibre size. **Other studies:** *in vitro* assembly assays showed assembly into a wider IF network; SW13 and MEF cells form a normal IF network.	[36,37]
c.137C>T **S46F**	?	**Skel:** MW**. Pathological studies:** abnormal myofibre size. **Other studies:** *in vitro* assembly assays showed IF assembly into wider filaments; SW13 cells form aggregates but MEF cells form a normal IF network.	[36,37]
c.137C>A **S46Y**	?	**Skel:** MW**. Pathology:** abnormal myofibre size. **Other studies:** *in vitro* assembly assays showed assembly into wider IF filaments; SW13 cells show aggregate formation but MEF cells form a normal IF network.	[36,37]
c.430A>T **K144X**	37 Card	**Card:** DCM; AVB.	[38]
c.640-2A>C ? (exon 3 skipping)	26-32 Card	**Card:** AVB that required pacemaker insertion.	[38]
c.1006G>T **D336Y**	37 Card	**Card:** DCM; AVB; pacemaker insertion.	[38]
c.1315G>A **E439D**	51 Card	**Card:** atrial fibrillation.	[38]
c.1325C>T **T442I**	27-35 Skel	**Skel:** MW and wasting leading to wheelchair dependence; MA. **Card:** atrial fibrillation and arrhythmia that required pacemaker insertion; fatal heart failure in some cases. **Pathology:** fibre splitting; internally located nuclei; Desmin-positive aggregates and vacuoles in myofibres. **Other studies:** *in vitro* assembly studies showed normal IF; SW13 and C2C12 cells form normal IF network.	[39]
c.1346A>C **K449Tx**	14 Skel	**Skel:** MW. **Pathology:** abnormal myofibre size. **Other studies:** *in vitro* assembly studies show normal filament formation; SW13 and C2C12 cells form normal IF network.	[36,39]
c.1360C>T **R454W** (+ Myotilin mutation)	15 Card	**Skel:** slowly progressive MW. **Card:** HCM that required Card transplantation. **Pathology:** Desmin-positive aggregates within myofibres. **Other studies:** *in vitro* assembly studies showed formation of short and irregular filamentous structures and aggregates; SW13 cells show aggregate formation and C2C12 form normal IF.	[39,40]
c.1379G>T **S460I**	29 Card	**Skel:** progressive MW and wasting. **Card:** AVB that required pacemaker implantation. **Pathology:** abnormal myofibre size; occasional split and regenerating fibres; vacuoles and Desmin-positive aggregates. **Other studies:** *in vitro* assembly studies showed normal filament formation; SW13 cells show aggregate formation but C2C12 form normal IF.	[39]
c.1413A>C **X471Y**	35 Card	**Card:** AVB that required pacemaker implantation.	[38]

'Other studies' describes results from animal models and *in vitro* systems. Skel: Skeletal muscle; Card: cardiac muscle; MW: muscle weakness; MA: muscle atrophy; DCM: dilated cardiomyopathy; HCM: hypertrophic cardiomyopathy; AVB: atrioventricular block; SW13 cells: human carcinoma cells; MEF cells: mouse embryonic fibroblasts; C2C12 cells: mouse myoblast/satellite cells.

Table 1. Description of clinical and pathological features of MFM caused by Desmin mutations.

Mutation		Classification	Ref
c.38C>T	**S13F**	DRM	[37,41-43]
c.46C>T*	**R16C**	RCM	[37,44]
c.322G>A	**E108K**	DCM	[45]
c.338A_339Gdel	**Q113fsX115**	DRM	[46]
c.517_537del*	**del(R173_179)**	DRM	[47,48]
c.638C>T (+α-glucosidase mutation)	**A213V**	DRM	[49,50]
c.639-1G>A + c.735+2A>G	**del(D214_E245)**	DRM	[44,51-53]
c.719dupA	**K240fsX243**	distal myopathy	[54]
c.735G>C/T	**E245D**	distal myopathy	[49,53,55]
c.893C>T	**S298L**	DCM	[45]
c.934G>A	**D312N**	DCM	[45]
c.1009G>C	**A337P**	DCM	[49,50,52,56,57]
c.1013T>G	**L338R**	DRM	[50]
c.1024A>G	**N342D**	DRM	[43,49,52]
c.1034T>C	**L345P**	DRM	[8,49,58,59]
c.1049G>C	**R350P**	distal myopathy and CM	[60]
c.1048C>T	**R350W**	DCM	[45]
c.1064C>G	**E355P**	DRM	[61]
c.1069G>C	**A357P**	DRM	[49,62]
c.1075_1083del	**del(E359_S361)**	DRM	[63]
c.1078G>C*	**A360P**	DRM	[49,52,56]
c.1097_1099del	**del(N366)**	DRM	[63,64]
c.1099A>T	**I367F**	DRM	[65]
c.1109T>C	**L370P**	DRM	[49,62,66]
c.1130T>C	**L377P**	DRM	[67]
c.1154T>C	**L385P**	DRM	[49,68]
c.1166A>C	**Q389P**	DRM	[49,69]
c.1175T>C	**L392P**	DRM	[65]
c.1178A>T*	**N393I**#	DRM	[49,50,52,56]
c.1195G>T	**D399Y**	DRM	[49,50]
c.1201G>A	**E401K**	DRM	[50]
c.1216C>T	**R406W**	DRM	[44,49,52,65,69,70]
c.1237G>A	**E413K**	DRM	[39,49,71]
c.1255C>T	**P419S**	DRM	[65]
c.1353C>G	**I451M**	DRM	[52,72-74]
c.1358C>T	**T453I**	DRM	[44]
c.1375G>A	**V459I**	DCM	[45]
c.1405G>A	**V469M**	DCM	[37,41-43]

All disorders are dominantly inherited unless otherwise indicated.*: autosomal recessive inheritance; DRM: Desmin-related myopathy; DCM: dilated cardiomyopathy; CM: cardiomyopathy; RCM restrictive cardiomyopathy.

Table 2. Human myopathies caused by Desmin mutations excluding MFM.

Mutations in Desmin result in many different myopathies (Table 1 and Table 2). Desmin-related myopathies (DRM) is a term that has been used to describe myopathies due to mutations in Desmin and CRYAB including MFMs, here we only use it to refer to those caused by Desmin mutations. In addition to the broad spectrum of DRM, Desmin mutations have also been classified as MFM, distal myopathy, DCM, and RCM (see Table 1 and Table 2). Some of the DRMs may be examples of MFM but without further information it is not

possible to re-classify them as MFMs. There is significant cardiac involvement in many Desmin myopathies and in some cases individuals with the same mutation may initially present with cardiac or skeletal muscle symptoms suggesting there is significant phenotypic variability and the possibility of modifiers of the Desmin myopathies. For example, the I451M mutation has been reported in a case of familial DCM without skeletal muscle phenotypes [72] and in individuals with skeletal myopathy without any evident cardiac defect [74]. Furthermore, the mutation was not fully penetrant in the family with DCM [72]. Potential modifiers include α-Glucosidase, with a single individual identified as a compound heterozygote for α-Glucosidase missense mutations and heterozygous for the Desmin A213V mutation displaying progressive muscle weakness not evident in related individuals carrying A213V alone [50], and Lamin A, as identified in an individual with Emery Dreifuss muscular dystrophy due to heterozygous Lamin A and Desmin V469M mutations [75].

Mutations in Desmin may also affect its capability to interact with its binding partners. Indeed, analysis of mutant protein E245D using solid phase binding assays showed that it binds to Nebulin with increased affinity, reducing Nebulin at the Z-disk, and is more prone to aggregate formation [76]. This interferes with Nebulin's ability to regulate the thin filament and results in disease [76]. Furthermore, the targeted mutation K190A, not yet observed in disease, shows decreased affinity for Nebulin resulting in decreased targeting of Nebulin to the Z-disk, its accumulation in aggregates in both skeletal and cardiac muscle, and narrower Actin bundles. It was therefore hypothesised that the lack of functional Desmin prevents Nebulin from stabilising Actin thin filaments thereby resulting in collapse of the contractile apparatus [21].

To study the role of Desmin in muscle function two independent knockout mice lines were created [77,78]. Both lines develop normally, are viable and fertile, with no defects in myogenesis. However, they present postnatal multisystem disorder, decreased myofibril alignment, defects in nuclear and mitochondrial positioning within the cell, and severe cardiac degeneration [77-81]. Muscle of *Desmin* knockout mice was also found to be more susceptible to damage following contraction [82]. These studies show that the absence of Desmin does not impair muscle formation or animal viability however, it is important for muscle function and integrity. In addition to the abnormal localisation of mitochondria described in the knockout mouse [81], Desmin mutations, such as K240fsX243, R350P, and E413K, can result in abnormal localisation and function of the mitochondria resulting in a deficiency in oxygen metabolism which impairs muscle function and may contribute to muscle degeneration [49,54,71].

It has been extensively suggested that Desmin may be essential in lateral force transmission by connecting adjacent sarcomeres, and even neighbouring myofibres, by costamere-extracellular matrix (ECM) binding. Therefore, Desmin mutations may impair its ability to respond to applied strain. Studies on Desmin with tail domain mutations in which the filament assembly is normal in both *in vitro* cell cultures and in transfected cells showed altered flexibility, with significantly increased stiffness compared to wildtype IF. This altered intrinsic properties of IF is hypothesised to prevent Desmin from responding to

excess strain thereby resulting in muscle pathology [40]. This is also supported by the del(Arg173-Glu179) knock-in mouse. Detailed analysis of the myocardium of these mice revealed the presence of aggregates containing Desmin and other muscle proteins, characteristic of desminopathies, which disturbed overall IF structure and compromised myocardium function both during baseline conditions and during maximal adrenergic stimulation [83].

The analysis of filament formation *in vitro* has identified a clear mechanism by which Desmin mutations may disrupt its assembly into filaments. However, it is still not clear which of the many roles of the IF contribute to pathology in desminopathies. The association of Z-disk proteins with MFM may suggest that it is the role of the IF at the Z-disk that is most relevant to these conditions but mitochondrial organisation and tethering of the myofibrils to the sarcolemma have clear links to muscle function and maintenance. The emerging application of whole genome and exome sequencing to mutation detection may improve identification of modifiers of pathology providing an alternative route to examine Desmin function, explain the phenotypic variations observed, and develop areas of potential therapy.

3. αB-crystallin and αB-crystallinopathies

To date 15 mutations in CRYAB have been reported. CRYAB belongs to the small Heat shock family of proteins (sHSP). It interacts with αA-Crystallin (CRYAA) via non-covalent bonds to form large heterogeneous macromolecular complexes [19]. Both CRYAA and CRYAB are found in high levels in the lens tissue of the eye where they are involved in maintaining lens transparency and refractive index [84]. CRYAB is also found in significant amounts in non-lenticular tissues such as skeletal and cardiac muscle, the kidney, and the brain [85-87]. In skeletal muscle CRYAB expression is highest in the oxidative slow twitch muscle and lowest in the glycolytic fast muscle [85,88]. In skeletal and cardiac muscle CRYAB is localised to the Z-disk [89] where it interacts with the I-band protein Actin [19] and various IF proteins including Desmin [19], Vimentin [90], and Glial fibrillary acidic protein (GFAP) [91].

The N-terminal globular domain and the highly conserved C-terminal 'α-crystallin domain' (ACD; Figure 2) of CRYAB are critical for its chaperone-like function [92-94] and dimerisation [95]. CRYAB prevents stress induced aggregation of various proteins including β- and γ-Crystallins [93,93], Desmin [19], Vimentin [90], and GFAP [91]. Following stressful conditions such as osmotic stress, metal toxicity [96], serum starvation, hypertonic stress, and heat shock [90] CRYAB expression is up-regulated and recruited to the IF to remodel the IF network [90]. Mutations in CRYAB have been shown to interfere with both its dimerisation and chaperone functions. Resolution of the crystal structure of the MFM causing R120G mutant protein showed a disruption to its tertiary structure predicted to interfere with its dimerisation and result in the formation of large soluble oligomers [97]. Moreover, the ACD domain of mutant CRYAB adopts an irregular structure, which decreases its chaperone function, makes it unstable and promotes its aggregation [98-100].

The mutant CRYAB has also been shown to have a higher dissociation constant, which prevents its dissociation from Desmin [101] resulting in Desmin containing aggregates as seen in MFM [100,101]. Therefore, alterations in the structure of CRYAB, its inability to perform its chaperone functions, and disruption of its interaction with its binding partners all contribute to disease pathology. In contrast to other MFM genes there does appear to be some correlation between genotype and phenotype with mutations in exon one resulting in isolated cataracts whilst exon three mutations can result in cataracts [102-107], MFM [108-110], distal myopathy [111], and/or CM [112,113], with two mutations resulting in both cataract formation and muscle failure [114,115] (Table 3).

Following contraction CRYAB is phosphorylated and translocates from the cytoplasm to the Z-disk which is thought to allow CRYAB mediated repair or protection of the Z-disk [125]. Recently, a rare case of infantile onset MFM was identified due to a homozygous frameshift mutation, S115fsX14, resulting in muscle stiffness [110]. The authors suggest the mutation results in a loss of contraction timulated translocation to the Z-disk and consequent reduction in muscle repair. CRYAB has also been implicated in indirectly preventing apoptosis and autophagy, inhibiting Caspase 3 mediated [126], Ras induced[127], and Bcl-2 mediated apoptosis [128]. It is therefore not surprising that CRYAB deficiency results in decreased cell viability and an increase in apoptosis in CRYAB knock-out mouse [129] and in patients suffering with CRYAB mutations [108]. In basal breast cancer CRYAB behaves as an oncoprotein [130] and in highly migratory glioma cells prevents apoptosis [131] making it a potential target in cancer therapy. Up-regulation of CRYAB may be part of a general protective mechanism since CRYAB is up-regulated in various pathological conditions such as cardiac ischemia [132], multiple sclerosis [133], Alzheimer's [134], and other neurodegenerative disorders [84].

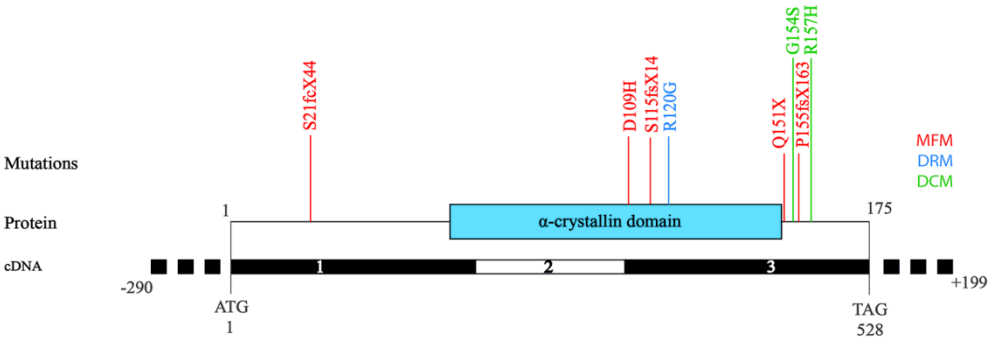
Wildtype CRYAB has the capability to bind to mutant protein to prevent its aggregation. For example, transfection of the MFM causing R120G mutant CRYAB into PtK2 cells results in the formation of aggregates but co-transfection with wildtype CRYAB, or the chaperone molecule Hsp27, results in a significant decrease in the amount of insoluble R120G CRYAB present in the cell and the frequency of aggregate formation [135]. Recently the BAG3 co-chaperone protein has also been shown to co-oligomerise with mutant CRYAB to suppress its aggregation and toxicity [136]. This does highlight that stimulating an increase in wildtype CRYAB, Hsp27, or BAG3 may be sufficient to prevent the formation of protein aggregates.

The observation that there is a 10 fold increase in CRYAB expression in differentiating and proliferating myoblasts [137,138] suggests that CRYAB may play a role in regulating myogenesis. CRYAB has been shown to decrease the synthesis, and increase the degradation, of MyoD, a myogenic regulatory factor that specifies cell lineage, resulting in delayed differentiation. Additionally, up-regulation of CRYAB in muscle cells results in sustained expression of cell cycle markers such as Cyclin D1 indicating cells were more proliferative [139]. Therefore, CRYAB can influence myogenesis by altering MyoD levels and cell cycle exit. Despite the potential for CRYAB to regulate muscle differentiation CRYAB knockout mice have normal muscle at birth but present with severe muscular dystrophy by week 40 suggesting CRYAB is not critical for muscle development but is essential for muscle function [129].

Mutation	Age of onset / Initial symptoms	Clinical and pathological features; other studies	Ref.
c.61delA* **S21fcX44**	Birth-11 weeks Resp	**Skel:** hypertonia. **Pathology:** presence of necrotic and regenerating fibres; atrophic and splitting myofibres and internally located nuclei; Desmin-, Myotilin-, and some Ubiquitin-positive aggregates at the periphery of myofibres, vacuoles and deposits. **Classified as MFM**	[9,109]
c.325G>C **D109H**	35-45 Skel	**Skel:** MW. **Card:** DCM; Cataract formation. **Pathology:** abnormal myofibre size; atrophic and splitting myofibres and internally located nuclei; Desmin-, CRYAB-, and Myotilin-positive aggregates and vacuoles. **Classified as MFM**	[114]
c.343delT* **S115fsX14**	4 months Skel	**Skel:** muscle stiffness. **Pathology:** muscle fibrosis. **Classified as MFM**	[110]
c.358A>G **R120G**	? Skel	**Skel:** MW; **Card:** HCM; Cataract formation. **Pathology:** presence of Desmin and CRYAB aggregates. **Other studies:** altered CRYAB quaternary structure; Partial unfolding exposes hydrophobic regions thus increases susceptibility to proteolysis and aggregation; disrupted protein binding; HeLa cells show hyperphosphorylation mutant CRYAB and accumulation in the cytoplasm; *in vitro* studies show Desmin and CRYAB aggregates in the cytoplasm and around the nuclei; rat cardiomyocytes with perinuclear aggregates containing Ubiquitin, β-Tubulin and Hsp25; cardiomyocytes in culture expressing mutant CRYAB show that the contractile apparatus does not work properly; mutant mice myofibrils alignment are impaired, CRYAB and in some cases Desmin- positive aggregates; cardiac hypertrophy; mitochondrial architecture and alignment are altered in cardiomyocytes; mice die by early adulthood. **Classified as DRM**	[98,99, 115-122]
c.451C>T **Q151X**	43 Skel	**Skel:** slowly progressive MW and MA. **Pathology:** severe abnormal myofibre size; necrotic and regenerating myofibres and internally located nuclei; abnormal Z-disks were detected; Desmin-, CRYAB- and Dystrophin-positive aggregates. **Other studies:** *in vitro* assays show that this mutation prevents oligomerisation, without changing its function, but aggregation is enhanced; *in vitro* assembly assays and COS-7 cells and cardiomyocytes cultures showed an increased tendency to hyperphosphorylation and aggregate formation. **Classified as MFM**	[108,123,124]
c.460G>A **G154S**	48-68 Skel or Card	**Skel:** slowly progressive MW and MA **Card:** DCM; moderate VEFR. **Pathology:** Desmin- and CRYAB-positive aggregates in subsarcolema and in the centre of the myofibres; Z-disk disorganization and smearing, with accumulation of vacuoles and other material. **Classified as DCM**	[111,112]
c.464CTdel **L155fsX163**	52 Resp	**Skel:** MW. **Pathology:** abnormal myofibre size; fibre degeneration; presence of vacuolations and inclusions; disruption of the intermyofibrillar architecture; Desmin-, Myotilin- and CRYAB-positive aggregates. **Classified as MFM**	[108]
c.470G>A **R157H**	40's Card	**Card:** ventricular tachycardia. **Other studies:** rat cardiomyocytes show decreased CRYAB binding to Titin in the cardiac specific domain, without affecting its distribution in the cell. **Classified as DCM**	[113]

'Other studies' describes results from animal models and *in vitro* systems. Mutations involved in isolated cataract formation: R11H; P20S; R56W; D140N; K150fsX184 and A171T are not shown. All disorders are dominantly inherited unless otherwise indicated. *: autosomal recessive inheritance; Skel: skeletal muscle; Card: cardiac muscle; Resp: respiratory system muscles; MW: muscle weakness; MA: muscle atrophy; DCM: dilated cardiomyopathy; HCM: hypertrophic cardiomyopathy; VEFR: ventricular ejection fraction reduction; MFM: myofibrillar myopathy; HeLa cells: human cervical cancer immortalised cells; COS-7 cells: African green monkey fibroblast.

Table 3. Description of clinical and pathological features of αB-crystallinopathies.



Mutations are coloured according to the disease classification. Dashed segments in cDNA represent the UTRs that are not drawn to scale.

Figure 2. Schematic representation of CRYAB protein structure and myopathy mutations.

4. Myotilin and myotilinopathies

The first description of myotilinopathy was a missense mutation in a family with LGMD1A [10]. Since this initial discovery, nine additional mutations in Myotilin (myofibrillar protein with Titin-like immunoglobulin domains) have been implicated in LGMD1A [10,140-142], MFM [143], SBM [144] or late onset distal myopathy [145] with all mutations described to date displaying an autosomal dominant pattern of inheritance. One of the mutations identified, S55F, has been found as a cause of both LGMD [141] and MFM [143] suggesting there may be modifiers of the disease that determine the symptoms produced or that there is an overlap in the classification of these conditions that needs to be resolved. Distinctions between these conditions are not clear, since the presence of protein aggregates is associated with MFM and SBM with weakness of distal muscle groups thought to be associated with MFM [143] and proximal muscle groups with LGMD (Table 4).

Myotilin belongs to the immunoglobulin domain containing Actin binding protein family that also contains the Actin organizing proteins Palladin and Myopalladin [146,147]. Myotilin is predominantly expressed in skeletal and cardiac muscle, with the highest levels present in the skeletal muscle. Expression is also detectable at low levels in the peripheral nerves, bone marrow, liver, thyroid gland and lung [153,154]. In skeletal muscle Myotilin is present in both slow type I and fast type II fibers [151] and is localised to the Z-disk [153], although some reports have suggested Myotilin may also be found at the sarcolemma [10,153,155]. A role at the sarcolemma is also supported by the inclusion of Dystrophin in the protein aggregates found in MFM and LGMD1A [10,143]. Like many other Z-disk proteins Myotilin is very dynamic as demonstrated by fluorescent recovery after photobleaching (FRAP) experiments in quail skeletal muscle that showed that 80% of Myotilin in the Z-disk is replaced within five minutes of bleaching [156].

Myotilin contains two identified domains, both essential for its function; a serine rich N-terminal domain, that shares no homology with any known protein, and a C-terminal domain consisting of two Ig-like domains that share high homology to two Z-disk associated Ig-like

domains of the giant protein Titin [153] (Figure 3). Seven of the eight identified Myotilin mutations, including the three MFM mutations [143], are in the serine rich domain with one mutation in the second Ig domain [142]. The serine rich domain consists of a stretch of hydrophobic residues that are believed to direct the localisation of Myotilin to the sarcolemma [10]. The serine rich domain is also responsible for the interaction of Myotilin with a range of proteins including the primary Z-disk crosslinker α-Actinin [153], Filamin- Actin- and Telethonin-binding protein of the Z-disk (FATZ, Myozenin, Calsarcin) [157], ZASP/Cypher [158], Filamin C [157,159] and the ubiquitin ligases MURF-1 and MURF-2 [160]. Interaction of Myotilin with FATZ directly or indirectly directs the localisation of FATZ to the Z-disk [157]. Myotilin also links Filamin C, found at the periphery of the Z-disk, to α-Actinin and anchors the Actin containing thin filaments to the Z-disk thereby providing stability to the sarcomere

Mutation	Age of onset / Initial symptoms	Clinical and pathological features; other studies	Ref
c.17G>A **R6H**	40 Skel	**Skel:** progressive MW, culminated in wheelchair dependence. **Pathology:** abnormal myofibre size and fibrosis; necrotic fibres with macrophage invasion; internally located nuclei; mitochondria aggregation. **Classified as LGMD1A**	[140]
c.116C>T **S39F**	Childhood-60s Skel	**Skel:** progressive MW, in some cases wheelchair dependence. **Pathology:** spheroid bodies with Myotilin immunoreactivity at the periphery. **Classified as spheroid body myopathy**	[144]
c.164C>T **S55F**	48-53 Skel	**Skel:** slowly progressive to severe MW and wasting. **Pathology:** abnormal myofibre size with deposits and vacuoles; atrophic and necrotic myofibres; Myotilin-, CRYAB-, Dystrophin-, Desmin-. and Ubiquitin-positive aggregates; clusters of mitochondria. **Classified as LGMD1A and MFM**	[141,143,148-150]
c.170C>T **T57I**	27 Skel	**Skel:** progressive MW**. Pathology:** abnormal myofibre size; myofibre degeneration and splitting; centrally located nuclei; vacuoles; Z-disc streaming. **Other studies:** mice reproduce human MFM pathology: Myotilin-, Desmin-, Ubiquitin-, and Actin-positive aggregates; fibrosis; Z-disk streaming and sarcomere disorganisation; some centrally located nuclei. **Classified as LGMD1A**	[10,151]
c.179C>G **S60C**	50-77 Skel	**Skel:** severe MW and wasting. **Card:** some asymptomic cases; DCM; VEFR; sometimes fatal**. Pathology:** abnormal and atrophic myofibres with deposits and vacuoles; Myotilin-, CRYAB-, Dystrophin-, Desmin- and Ubiquitin-positive aggregates. **Classified as MFM**	[143,148,152]
c.179C>T **S60F**	40-76 Skel	**Skel:** difficulty in walking and climbing stairs; MW**. Classified as distal myopathy**	[145]
c.284G>T **S95I** ?	?	**Pathology:** abnormal and atrophic myofibres with deposits and vacuoles; Myotilin-, CRYAB-, Dystrophin-, Desmin- and Ubiquitin- positive aggregates **Classified as MFM**	[143]
c.1214G>A **R405K**	41 Skel	**Skel:** impossibility to walk long distances; MW**. Pathology:** abnormal myofibre size; scattered fibres with internally located nuclei; vacuoles and Myotilin-, ZASP-, Desmin- and Actin-positive aggregates. **Classified as LGMD1A**	[142]
c.1214G>A **R405K**	41 Skel	**Skel:** impossibility to walk long distances; MW**. Pathology:** abnormal myofibre size; scattered fibres with internally located nuclei; vacuoles and Myotilin-, ZASP-, Desmin- and Actin-positive aggregates. **Classified as LGMD1A**	[140]

K36E and Q74K mutations are not shown since no information is available (shown in [142]). 'Other studies' describes results from animal models and *in vitro* systems. Skel: Skeletal muscle; Card: cardiac muscle; MW: muscle weakness; DCM: dilated cardiomyopathy; VEFR: ventricular ejection fraction reduction; MFM: myofibrillar myopathy.

Table 4. Description of clinical and pathological features of myotilinopathies.

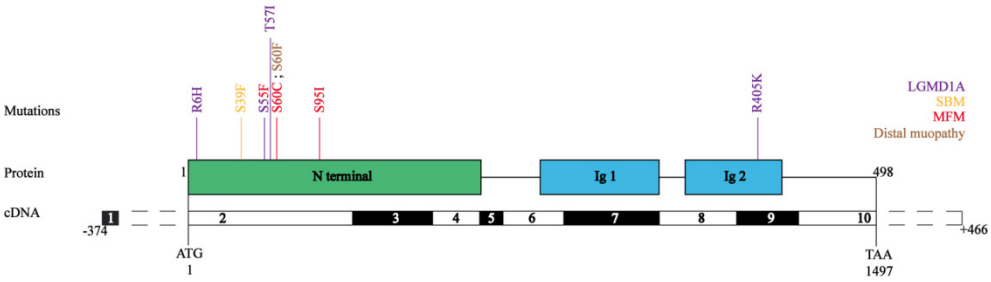
[159,161]. The Ig domain containing C-terminus on the other hand is responsible for antiparallel dimerization of Myotilin, which is essential for its function [153,161]. The Myotilin C-terminus also interacts directly with Actin, despite the lack of a conventional Actin binding site [162], and is thought to prevent the depolymerisation of Actin filaments and enhance the binding of α-Actinin to Actin. Overexpression of Myotilin in CHO cells results in formation of Actin bundles and the delayed expression of Myotilin relative to other Z-disk proteins is thought to be required to avoid premature bundling of Actin fibres [161,162].

Despite the loss of muscle integrity in myotilinopathies Myotilin knockout mice display no muscle defects. Their Z-disk structure and sarcolemma integrity is maintained with no effect on muscle strength and the heart appears normal [163,164]. This suggests that other closely related proteins, such as Palladin and Myopalladin, may have overlapping functions to that of Myotilin and are able to compensate for its loss. Interestingly, mice deficient in Myotilin have a two fold increase in the expression of the muscle stretch sensor Telethonin, which may be responsible for sensing the lack of Myotilin and triggering appropriate signals to prevent muscle failure [163]. However, this hypothesis needs to be validated.

Analysis of Myotilin levels in patients compared to control individuals failed to identify a reduction in protein level [10,142] with other studies reporting an increase in Myotilin in some patients [142,165]. This observation leads to the hypothesis that mutations in Myotilin affect its dimerisation or interaction with binding partners, resulting in pathology. This is certainly true in the case of the identified missense mutation affecting the second Ig domain of Myotilin (R405K) resulting in LGMD [142]. The R405K mutation prevents dimerisation disrupting α-Actinin binding and Actin tethering to the Z-disk. As a result, filament stability is lost, the Z-disk is destabilised, and protein aggregates are formed [142]. However, this is not true for the majority of Myotilin mutations, which are found in the serine rich N-terminal domain. *In vitro* experiments have shown that the S55F, T57I, S60C, and S95I, mutations have no effect on Actin bundling [162], and T57I also has no effect on the interaction with α-Actinin [10]. It has since been hypothesised that mutated Myotilin promotes intermolecular aggregation with other similar Ig domain containing sarcomeric proteins such as Palladin and Titin. The mutant Myotilin expressing transgenic mouse (T57I) that contains aggregates rich in Ig domain containing proteins [151] supports this hypothesis. Interestingly, although in humans the T57I mutation results in LGMD1A in which aggregates are absent, expression in mice results in formation of aggregates that are typical of MFM and SBM. This led to the authors suggesting that the variation in symptoms between MFM, SBM, and LGMD1A, such as protein aggregates, are due to modifying loci [151].

The formation of protein aggregates is a defining feature of MFM and SBM. Aggregate containing muscle from myotilinopathy patients contains increased levels of oxidative stress markers including glycation end products, nitric oxide synthase, superoxide dismutase, and mutant Ubiquitin [166,167]. Protein oxidation promotes protein aggregation and reduces proteolytic degradation. Another factor that may promote protein aggregation in myotilinopathies and other MFMs is the inability of cells to degrade misfolded proteins via

the non-lysosomal ubiquitin proteasome system (UPS) responsible for the degradation of 80-90% of myofibrillar proteins [168]. UPS mediated degradation of mutant Myotilin is significantly slower than wildtype Myotilin and inhibition of Myotilin turnover results in aggregates similar to those seen in MFM [169].



Mutations are coloured according to the disease classification. Dashed segments in cDNA represent the UTRs that are not drawn to scale.

Figure 3. Schematic representation of Myotilin domains and myopathy mutations.

5. ZASP and zaspopathies

Z band alternatively spliced PDZ-containing protein (ZASP) [170], also known as LIM Domain Binding 3 (LDB3), Cypher [171], or Oracle [172] belongs to the PDZ-LIM family of proteins and similar to other members of the family, Enigma [173] and α-Actinin associated LIM protein (ALP) [174], localises to the Z-disk. More than 15 mutations in ZASP have been reported resulting in a range of myopathies including DCM [11,175], HCM [176], MFM [177], inclusion body myositis [178], and LVNCC [11,175,177] (Table 5). ZASP contains a PDZ domain, located at the N-terminus, and an internal ZASP/cypher-like motif (ZM) both capable of interacting with α-Actinin-2 [179-181]. Additionally, the PDZ domain interacts with Myotilin [158] and FATZ [182], which provides structural stability to the Z-disk. The C-terminus contains three LIM domains, which act to recruit signalling proteins to the Z-disk.

As its name suggests the ZASP mRNA is extensively spliced to result in multiple ZASP isoforms, a feature conserved in all species examined with four isoforms in worms [183], 12 isoforms in flies [184-186], 13 in zebrafish [187], and six in mice and humans [11,188] (Figure 4). In mice and humans the isoforms have been characterised according to their length and their expression in the heart or skeletal muscle. So far two short isoforms, (2c, 2s) that lack the LIM domains, and four long isoforms (1c, 1s, 3c and 3s), that contain all three LIM domains, have been characterised [11,170]. Isoforms containing exon four (1s, 2s and 3s) are restricted to cardiac muscle whereas isoforms lacking exon four are found in both cardiac and skeletal muscle [11]. Loss and gain of function experiments have highlighted specific roles for the short and long isoforms. Selective deletion of the short isoforms does not lead to any muscle defects however, loss of the long isoforms results in neonatal lethality in 28% of mice [189]. Surviving knockout mice display growth retardation and Z-disk abnormalities in cardiomyocytes leading to DCM in adulthood, demonstrating the requirement for the

Mutation	Age of onset / Initial symptoms	Clinical and pathological features; other studies	Ref
c.163G>A* **V55I**	? Card	**Card:** LVNCC. **Classified as LVNCC**	[175,191]
c.349G>A **D117N**	30-41 Card	**Card:** DCM; AVB; EFR; premature death in some cases. **Other studies:** C2C12 show ZASP aggregates formation and abnormal Actin staining. **Classified as LVNCC**	[11]
c.407C>T **K136M**	16 Card	**Card:** DCM; VEFR. **Classified as LVNCC**	[11]
c.464G>A (mRNA) **A147T**	44-59 Skel	**Skel:** progressive MW and MA. **Card:** some cases of VEFR. **Pathology:** atrophic, necrotic, and regenerating myofibres; fibre splitting and internally located nuclei; small vacuoles and Desmin-, Myotilin-, CRYAB-, and Dystrophin-positive aggregates; streaming and disintegration of the Z-disk; organelles in clusters. **Classified as MFM**	[177]
c.519C>T (mRNA) **A165V**	39-59 Skel	**Skel:** progressive MW and MA. **Pathology:** atrophic, necrotic, and regenerating myofibres; fibre splitting and internally located nuclei; small vacuoles and Desmin-, Myotilin-, CRYAB- and Dystrophin-positive aggregates; streaming and disintegration of the Z-disk; organelles in clusters. **Classified as LVNCC**	[177,192]
c.587C>T **S196L**	7-73 (not accurate) Card	**Card:** DCM or HCM; VEFR. **Other studies:** mice show left ventricular dilation; HCM, VEFR; mild focal fibrosis; sarcomere and Z-disk disorganisation. **Classified as DCM**	[11,176,193]
c.638C>T **T213I**	15 months Card	**Card:** AVB; VEFR. **Other studies:** Reduced binding to PGM1 **Classified as DCM and LVNCC**	[11]
c.827C>T (mRNA) **R268C**	73 Skel	**Skel:** progressive MW. **Pathology:** atrophic, necrotic, and regenerating myofibres; fibre splitting and internally located nuclei; small vacuoles and Desmin-, Myotilin-, CRYAB-, and Dystrophin-positive aggregates; streaming and disintegration of the Z-disk; organelles in clusters. **Classified as MFM**	[177]
c.1056C>G **I352M**	<15-35 Card	**Card:** DCM; VEFR. **Other studies:** Reduced binding to PGM1. **Classified as DCM**	[11]
? **D366N**	68 Card	**Card:** HCM. **Classified as HCM**	[176]
? **Y468S** (+CRSP3 mutation)	46 Card	**Card:** HCM. **Classified as MFM**	[176,191]
? **Q519P**	21 Card	**Card:** HCM. **Classified as HCM**	[176,191]
c.1719G>A **V566M**	40 Skel	**Skel:** slowly progressive MW; MA. **Pathology:** abnormal myofibre size; vacuoles and Desmin-, Myotilin-, CRYAB- and Ubiquitin-positive aggregates	[178]
? **P615L**	28 Card	**Card:** HCM. **Classified as HCM**	[176]
c.1876G>A **D626N**	after birth-69 Card	**Card:** DCM; LVNC. **Other studies:** mice show that mutant ZASP has higher affinity to PKC, which may cause the heart failure. **Classified as LVNCC and DCM**	[175,191]

All conditions are dominantly inherited unless otherwise indicated. 'Other studies' describes results from animal models and *in vitro* systems. *: autosomal recessive inheritance; Skel: Skeletal muscle; Card: cardiac muscle; LVNCC: left ventricular non compaction cardiomyopathy; DCM: dilated cardiomyopathy; HCM: hypertrophic cardiomyopathy; AVB: atrioventricular block; VEFR: ventricular ejection fraction reduction; MW: muscle weakness; MA: muscle atrophy; MFM: myofibrillar myopathy; C2C12 cells: mouse myoblast/satellite cells

Table 5. Description of clinical and pathological features of zaspopathies.

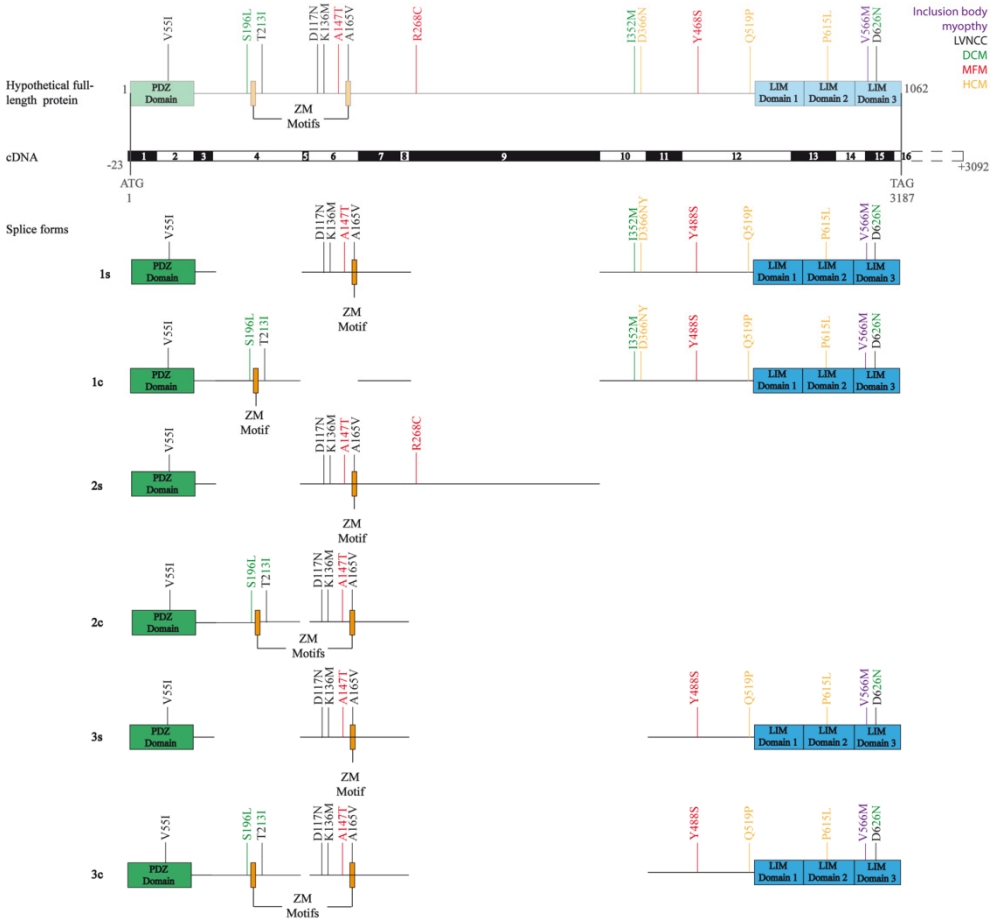
long, but not the short, isoforms in maintaining Z-disk integrity [189]. Loss of both short and long isoforms however, results in death within the first five days [190] suggesting that there is some

redundancy in their roles. This hypothesis is supported by rescue experiments showing that expression of either the short or long skeletal isoform in ZASP deficient mice is sufficient for survival in 19% and 49% of carriers respectively [188]. The different phenotypes observed following loss of long or short isoforms raises the question whether mutations in specific isoforms result in specific myopathies. This is clearly true in the case of mutations in the cardiac specific exon four however, why mutations in exons expressed in both skeletal and cardiac muscle results in only one tissue getting affected is not clear. For example the D117N [11], A147W, and A165V [177] mutations in exon six affect both skeletal and cardiac muscle isoforms. However, D117N preferentially affects the cardiomyocytes whereas A165V preferentially affects the skeletal muscle, and A147W results in both tissues being affected. Therefore, there appears to be no clear correlation between the exon affected and the phenotype presented by the patient.

Examination of the diaphragm muscles, which are not active before birth, in ZASP knockout mice identified little or no difference in the sarcomere structure at E17.5 when compared with wildtype mice, but severe disruption of the Z-disk the day after birth [190], suggesting that ZASP is not required for sarcomere assembly but is critical for maintenance of Z-disk integrity. Examination of cardiac muscle in these mice, which becomes active at E8, at E17.5 identified severely disrupted Z- disks which were completely lost by one day after birth [190]. A role for ZASP in Z-disk maintenance is supported by experiments demonstrating that deletion of ZASP in postnatal hearts results in gradual disruption of the Z-disk and severe DCM resulting in premature death within five months [194]. Targeted deletion of ZASP homologues in *Drosophila* results in defects in muscle development suggesting a role for ZASP is in *Drosophila* sarcomerogenesis [184,185]. However, *Drosophila* has only a single protein equivalent to the mammalian ZASP, ALP, and Enigma proteins. It is therefore possible that in mammals, ZASP, ALP, and Enigma have redundant roles and loss of all three proteins in mammals would result in a phenotype similar to that seen in *Drosophila*. In *Drosophila* ZASP was identified as a regulator of cell matrix adhesion localising to integrin adhesion sites in S2 and S2R+ cell lines co-localising with α-Actinin at the Z-disks and integrins at the myotendinous junctions in embryos [184]. ZASP deficient flies display a muscle detachment phenotype and lack α-Actinin at the Z-disk, suggesting that the interaction of ZASP with Integrin is critical in connecting the muscle fibre to the ECM and in directing α–Actinin to the Z-disk [184]. However, localisation of ZASP to myotendinous junctions or costameres has not been reported in any other animal model.

In cardiomyocytes ZASP interacts with Protein Kinase C (PKC) [175], a known modulator of cardiomyocyte growth and contractility. PKC-ε has been shown to interact with RACK-2 and protect cardiomyocytes from ischemic stress [195,196]. Disruption of the PKC-ε - RACK-2 complex results in inhibition of cell contraction [197] and accelerated cell death [198]. *In vivo* studies have revealed increased levels of PKC in hypertrophy, DCM, and heart failure [199-201], suggesting a role for PKC in stress response, potentially modulated by ZASP. Biochemical analysis of ZASP revealed that the D626N LIM domain mutation increases the binding affinity of ZASP for PKC. The authors suggest that this may reduce the amount of PKC-ε available to bind downstream proteins such as RACK-2 therefore resulting in DCM due to altered distribution of PKC [175]. ZASP also interacts with the metabolic protein Phosphoglucomutase 1 (PGM1), an enzyme involved in glycolysis and gluconeogenesis,

through the proline rich regions encoded by exons four, six and, ten and recruits it to the Z-disk [202]. DCM causing mutations in exon four (S196L and T213I) and exon ten (I352M) have been shown to have reduced binding affinity for PGM1 [202]. The binding of ZASP to PGM1 and ZASP mediated targeting of PGM1 to the Z-disk are both increased under stress condition further supporting a role of ZASP in protection and repair of the Z-disk, although the role of PGM1 at the Z-disk is not clear [202].



ZASP contains 16 exons, although no ZASP protein is coded by the hypothetical full-length cDNA. The hypothetical full-length protein is a representation of all protein domains and all mutations described in humans so far. Six splice forms have been described (1s, 1c, 2s, 2c, 3s, 3c) and named accordingly to the presence specific exons, such as the cardiac specific exon 4 (c for cardiac and s for skeletal). Each splice form is shown with all mutations present on the exons it contains accordingly to the amino acid change described when published. Therefore, numbering incongruences are detected depending on the splice form analysed. Mutations are coloured according to the disease classification. Note that the 3'UTR is not drawn to scale.

Figure 4. Schematic representation of *ZASP* domains, human splice variants, and mutations.

6. Filamin and filaminopathies

Filamin C (FLNC) was first implicated in MFM in 2005 with the identification of a missense mutation in a German family that presented with weakness of the proximal muscle groups and respiratory insufficiency [12]. Since this initial discovery five additional FLNC mutations have been identified of which two result in MFM [203,204] and three cause distal myopathies in which protein aggregates are not evident [205,206] (Table 6).

Mutation	Age of onset / Initial symptoms	Clinical and pathological features; other studies	Ref
c.577G>A **A193T**	30's Skel	**Skel:** MW. **Pathology:** residual fibre size variation; focal increase in fibrosis and internal nuclei. **Other studies:** C2C12 show increased number of stress fibres and cell projections; FLNC- and Actin-positive aggregates were detected. **Classified as distal myopathy**	[205]
c.752T>C **M251T**	30's Skel	**Skel:** slowly progressive MW. **Card:** some developed CM. **Pathology:** abnormal myofibre size; internally located nuclei. **Classified as distal myopathy**	[205]
c.2695_2712del + GTTTGTins **del(K899_V904) + ins(V899_C900)**	35-40 Skel	**Skel:** progressive MW. **Card:** AVB. **Pathology:** variation in myofibre size and increased numbers of internal nuclei; vacuoles and deposits positive for Desmin, Dysferlin, Dystrophin and Ubiquitin; necrotic and regenerating myofibres; nemaline bodies. **Classified as MFM**	[204]
c.2788_2799del **del(V930_T933)**	34-60 Skel	**Skel:** difficulty to stand or walk; progressive MW. **Pathology:** abnormal myofibre size; atrophic myofibres and internally located nuclei; aggregates positive for FLNC, Ubiquitin, Desmin, Myotilin and CRYAB; nemaline bodies and mitochondria aggregates. **Classified as MFM**	[203]
c.5160delC **F1720fsX633**	20-57 Skel	**Skel:** MW and MA. **Card:** few cases of CM and VEFR. **Pathology:** from slight myofibre size variation and rare fibre splitting and internally located nuclei to myofibrillar disorganisation, Z-disk streaming, presence of small rods and other deposits. **Classified as distal myopathy**	[206]
c.8130G>A **W2710X**	24-49 Skel	**Skel:** slowly progressive MW; wheelchair dependence in some patients. **Card:** some patients with HCM, AVB and VEFR. **Pathology:** splitting and necrotic fibres; internally located nuclei; aggregates positive for FLNC, Desmin, Myotilin and Dystrophin and vacuoles; Z-disk streaming and nemaline-rod formation. **Other studies:** protein studies showed a decreased stability and dimerisation capacity of the mutant FLNC; PtK2 cells form aggregates. **Classified as MFM**	[4,12,207]

'Other studies' describes results from animal models and *in vitro* systems. Skel: Skeletal muscle; Card: cardiac muscle; MW: muscle weakness; MA: muscle atrophy; CM: cardiomyopathy; HCM: hypertrophic cardiomyopathy; AVB: atrioventricular block; VEFR: ventricular ejection fraction reduction; MFM: myofibrillar myopathy; C2C12 cells: mouse myoblast/satellite cells; PtK2 cells: Potorous tridactylis kidney cells.

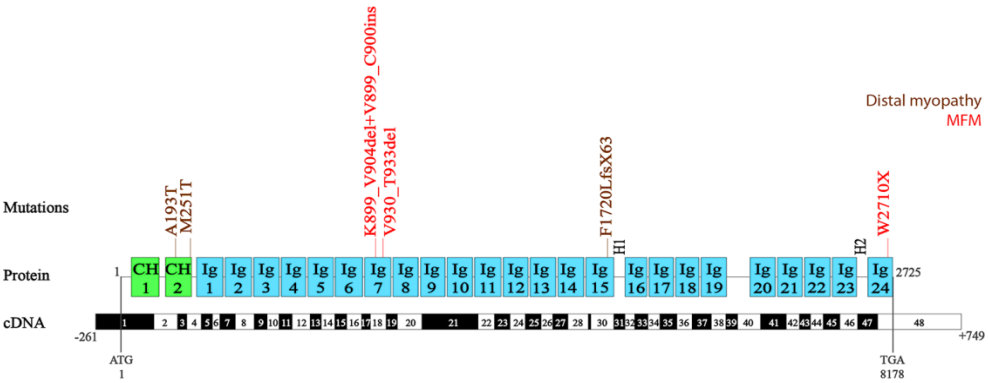
Table 6. Description of clinical and pathological features of filaminopathies.

FLNC belongs to the Filamin family of proteins characterised by their ability to cross link Actin. Three Filamin isoforms have been identified all of which are encoded by different genes [208,209]: Filamin A (α-Filamin or Filamin 1) and Filamin B (β-Filamin), which are ubiquitously expressed, and FLNC (Filamin 2, γ-Filamin, Actin Binding Protein 280 (ABP-280) or Actin Binding Protein Ligand (ABP-L)) [210-212], which is expressed specifically in striated and cardiac muscle [204,212]. In striated muscle, FLNC localises in two different pools: 97% of FLNC is contained within the Z-disk of the sarcomere and 3% is found in the

sarcolemmal membrane at the level of the costameres and myotendinous junctions [213]. In cardiac muscle FLNC is found in intercalated discs [213,214].

Filamin proteins contain two distinct functional regions (Figure 5). The N-terminal region, which contains two calpain homology domains that are responsible for interacting with Actin and promoting its polymerisation [215]. Two of the three distal myopathy causing FLNC mutations, in which protein aggregates are not evident, are found in this N-terminal region. This suggests that the presence of a functional N-terminal Actin binding domain in MFM causing FLNC mutant protein may be important in the formation of protein aggregates. The other four FLNC mutations are found in the semi-flexible rod domain, which contains 24 homologous Ig-like domains, each about 93 to 103 amino acids long [203]. The Ig-like domains act as an interface for the interaction of FLNC with its binding partners and allow FLNC dimerisation, through domain 24 [216], which is essential for its function.

Disruption of FLNC dimerisation leads to failure of the Z-disk as in the case of W2710X MFM causing FLNC mutation. Truncation of the dimerisation domain results in the loss of secondary structure of the mutant protein hence making it less stable and more susceptible to degradation by proteolytic enzymes [12,207], but is also more prone to aggregation [207] . Although the mutant protein is unable to form dimers, it neither disturbs dimerisation of wildtype FLNC nor affects its interaction with Actin or the sarcoglycans, two key FLNC binding partners [207]. In between FLNC Ig-like domains 15 and 16 (Figure 5), a differentially spliced Hinge 1 (H1) region is present, that provides flexibility to FLNC, but is absent from the predominant form expressed in striated muscle. Additionally, FLNC has a second hinge region (H2, Figure 5) between Ig-like domains 23 and 24, found in both splice variants [210,212], and contains a unique 82 amino acid insert between Ig-like domains 19 and 20 [213], which is thought to recruit FLNC specifically into the Z-disk [157].



Mutations are coloured according to the disease classification.

Figure 5. Schematic representation of FLNC domains and mutations.

FLNC has been proposed to have several functions in the muscle. The interaction of Ig-like domain 20 of FLNC with the Z-disk protein Xin is important in regulating the development

and remodelling of the Actin cytoskeleton [217]. Additionally, the interaction of FLNC Ig-like domains 19, 20, 21, and domain 23 with the Z-disk proteins Myotilin [159] and FATZ [157,182,218,219] maintains the stability of the sarcomere. At the sarcolemma, FLNC interacts with the transmembrane proteins γ- and δ-Sarcoglycans (repeats 20 to 24) [213], Cbl-associated protein (CAP or Ponsin, domain 2) [220], Ankyrin G (repeat 5 and 6) [221], and β1-integrin (domain 20-21) [157]. FLNC therefore connects the Z-disk to the sarcolemma and the ECM providing both a structural linkage and a mechanism for signalling from the sarcolemma to the Z-disk [159,213]. In cardiac muscle FLNC interacts with Nebulette [222], the cardiac specific homologue of the thin filament ruler Nebulin. This interaction has been thought to be important in targeting FLNC to the cytoskeleton therefore ensuring the correct localisation and function of FLNC. FLNC, through Ig-like domains 20, 21, and 23 also interacts with the muscular dystrophy KY protein but the functional importance of this interaction is not known [214]. The identification of a distal myopathy as a result of FLNC haploinsufficiency suggests that the levels of FLNC maybe critical for its function [206]. Additionally, the altered distribution of both sarcomeric and ECM proteins in filaminopathies suggests that the functions of FLNC at the Z-disk and sarcolemma are compromised in filaminopathies. Analysis of the FLNC mouse knockout identified a decrease in the number of primary muscle fibres suggesting a role for FLNC in myogenesis [223]. However, the recent characterisation of a Medaka FLNC mutant showed no difference in the expression of myogenic factors [224]. The role of FLNC in fibre differentiation is therefore still questionable.

The process by which mutations in FLNC result in muscle disease is not understood but the identification of a haploinsufficient form of filaminopathy, and the finding that the W2710X mutant does not disrupt wildtype FLNC dimerisation, together with the severe muscle defects seen in the FLNC knockout mouse suggest that Filamin related MFM manifests as result of direct or indirect loss of functional FLNC. It is therefore hypothesised that the progressive, late-onset, nature of filaminopathies results from a reduction in FLNC function commensurate with the increasing sequestration of wildtype FLNC and FLNC binding partners by mutant FLNC in the cytoplasm.

7. BAG3 and bag3opathies

Bcl2-related athanogene 3 (BAG3, Bis, CAIR) is the most recently identified MFM causing Z-disk protein with the report of a missense mutation (P209L) in exon three resulting in MFM with cardiac complications [13]. Since then 10 additional *Bag3* mutations have been reported of which nine result in DCM [225,226] and one in MFM [227] (Table 7). BAG3 is one of six members of the BAG family of proteins. It is a multidomain co-chaperone expressed at high levels in skeletal and cardiac muscle and found at lower levels in tissues such as neurons, adrenal gland, ovaries and testis [228,229]. In skeletal muscle BAG3 co-localises with Desmin and α-Actinin at the Z-disk [228]. An increase in BAG3 expression is detected following an increase in static strain [230], eccentric contraction [231], or nemaline myopathy [232] which suggests that BAG3 plays a role in repair and regeneration of skeletal muscle injuries caused by mechanical stress and disease.

Mutation	Age of onset / Initial symptoms	Clinical and pathological features; other studies	Ref
c.211C>T **R71W**	41-59 Card	**Card:** DCM; VEFR; heart transplantation required. **Classified as DCM**	[225]
c.268C>T **R90X**	44 Card	**Card:** DCM; VEFR. **Classified as DCM**	[225]
c.326A>G **H109R**	21 Card	**Card:** DCM; VEFR. **Classified as DCM**	[225]
c.367C>T **R123X**	25-36 Card	**Card:** DCM in some cases; some cases with VEFR; some required heart transplantation. **Classified as DCM**	[225]
c.626C>T **P209L**	5-13 Skel and Card	**Skel:** moderate to severe MW and MA; easy fatigability **Card:** Restrictive HCM; heart transplantation needed in some cases; early death in most cases. **Pathology:** abnormal myofibre size which larger fibres showed splitting or breakdown; necrotic and regenerating myofibres and internally located nuclei; abnormal fibres with ectopic staining for BAG3, CRYAB, Desmin, Myotilin, Dystrophin and Ubiquitin; presence of dense structures and aggregates of mitochondria; Z-disk streaming. **Other studies:** neonatal rat cardiomyocytes showed problems in cell fusion; COS-7 cells presented granules in their cytoplasm; C2C12 with reduced BAG3 protein levels show increased apoptosis. **Classified as MFM**	[13,226, 228,240]
c.625C>T + c.772C>T **P209W + R258W**	6 Skel	**Skel:** progressed from clumsy walking into MW and decreased spine movement **Card:** restrictive HCM. **Pathology:** atrophic fibres; focal myofibrillar disorganisation and degeneration; sarcoplasmic accumulation of granulofilamentous material. **Classified as MFM**	[227]
c.652C>T **R218W**	73 Card	**Cardiac muscle:** ventricular wall thickness; severe VEFR; ectopic atrial rhythm**. Other studies:** neonatal rat cariomyocyes presented abnormal Z-disk assembly (seen by Desmin and α-Actinin staining) and increased susceptibility to apoptosis. **Classified as DCM**	[226]
c.652Cdel **R218fsX89**	47 Card	**Card:** DCM; VEFR; early death. **Classified as DCM**	[225]
c.784G>A **A262T**	42-44 Card	**Card:** DCM; AVB; severe VEFR; required heart transplantation. **Classified as DCM**	[225]
c.1385T>C **L462P**	27-34 Card	**Card:** ventricular wall thickness; VEFR; cardiac contraction defects. **Other studies:** neonatal rat cardiomyocytes presented abnormal Z-disk assembly and increased susceptibility to apoptosis. **Classified as DCM**	[226]
c.1430G>A **A477H**	47-50 Card	**Card:** DCM; severe VEFR; pacemaker insertion. **Classified as DCM**	[225]

'Other studies' describes results from animal models and *in vitro* systems. Skel: Skeletal muscle; Card: cardiac muscle; MW: muscle weakness; MA: muscle atrophy; HCM: hypertrophic cardiomyopathy; DCM: dilated cardiomyopathy; VEFR: ventricular ejection fraction reduction; AVB: atrioventricular block; MFM: myofibrillar myopathy; COS-7 cells: African green monkey fibroblast; C2C12 cells: mouse myoblast/satellite cells.

Table 7. Description of clinical and pathological features of bag3opathies.

BAG3 has three recognisable functional domains (Figure 6); no mutations have been reported in the WW domain containing N-terminal region, which interacts with proline rich motifs of signal transduction proteins, or in the proline rich central region, which interacts with WW domains and Src3 homology (SH3) domains of signal transduction proteins such as Phospholipase C (PLCγ) [233].

Two mutations have however been reported in the evolutionary conserved C-terminal BAG domain that has a key role in the apoptotic and chaperone functions of BAG3 [234-236]. The BAG domain binds with high affinity to, and regulates, stress inducible Heat shock protein 70 (Hsp70) [233-235,237] and the constitutively expressed Heat shock cognate protein 70 (Hsc70) [237], that ensure

correct protein folding and targeting of misfolded proteins for proteasomal degradation [238]. BAG3 competitively binds to the ATPase domain of these chaperone proteins and alters their chaperone properties thereby targeting chaperone associated proteins for proteasomal degradation [237,239]. In fact, BAG3 has recently been shown to form a stable complex with the small Heat shock protein HspB8 and stimulate macroautophagy [236], a process that is particularly important in Huntington disease where association of BAG3 with HspB8 promotes degradation of mutant Huntingtin [236,241]. In inclusion body myositis, macroautophagy plays a role in removing β-amyloid aggregates [242] and it is possible that BAG3 also plays a role in this cellular response to protein aggregates in MFM. However, as the primary defect in MFM is the dissolution of muscle fibres beginning at the Z-disk preventing the formation of protein aggregates is unlikely to be sufficient to prevent muscle pathology.

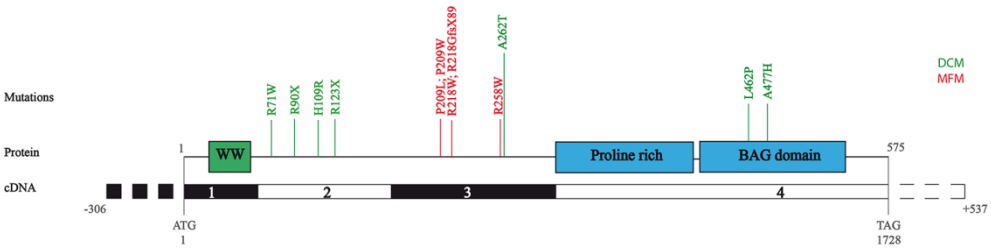
In αB-crystallinopathies BAG3 suppresses protein aggregation and toxicity by preferentially binding mutant CRYAB, reducing its aggregation and increasing its solubility [136]. This demonstrates that BAG3 not only indirectly regulates protein folding and degradation but also has the potential to prevent misfolding and promote degradation of mutant proteins thereby preventing disease pathology. Interestingly, deletion of the BAG domain results in a similar inhibition of aggregation of mutant CRYAB. BAG3 may therefore function through a pathway that is independent of Hsp70/Hsc70 [136]. BAG3 synergistically interacts with Bcl-2, via the BAG domain, to prevent Bax induced and FasL-

Fas mediated apoptosis [239,243]. BAG3 levels are up-regulated in response to oxidative stress [244], heat shock, heavy metal exposure [245,246], or photoinjury in the retina [247] and increased levels of BAG3 in human epithelial cells has been show to result in decreased Bax or Fas mediated apoptosis demonstrating the critical adaptive role of BAG3 in response to cell stress.

Immunohistochemistry on P209L mutant muscle biopsy samples revealed increased immunoreactivity in abnormal fibres for the chaperone molecules Hsp27 and CRYAB and the anti- apoptotic protein Bcl-2. This was accompanied by increased apoptosis suggesting that the P209L mutation interfered with the anti-apoptotic functions of BAG3 [13]. Non-denaturing gel electrophoresis revealed faster migration of the mutant BAG3 complex than wildtype [13], suggesting that the loss of function may be due to reduced interaction with partner proteins, possibly Bcl-2, given that an increase in apoptosis is observed. This is supported by transfection of P209L mutant BAG3 into neonatal cardiomyocytes resulting in increased susceptibility to stress mediated apoptosis [226] and the observation that mice deficient in BAG3 also display increased apoptosis [228]. It has been shown that the down-regulation of BAG3 enhances the apoptotic response to chemotherapy in lymphocytic

leukaemia cells making it a potential target for cancer therapies [248], and further demonstrating its anti-apoptotic role.

Since the primary defect in BAG3opathies is the fragmentation of fibres it can be postulated that perhaps BAG3 has a role in muscle development or maintenance of muscle structure. BAG3 deficient mice are normal at birth but cease to gain weight at day 12. Muscle histology revealed myofibril and Z-disk defects with no sarcolemma damage [228]. Taken together, this data suggests that BAG3 is not necessary for sarcomerogenesis but is critical for maintenance of fibre integrity. By the 25th day BAG3 deficient mice die as a result of intercostal muscle failure or pulmonary oedema that results in cardiac failure [228]. Targeted knockdown of BAG3 in zebrafish has also resulted in severe cardiac defects demonstrating a critical role of BAG3 in maintaining the structural integrity of cardiomyocytes [225]. A recent study has shown that BAG3 regulates myofibril stability by facilitating the interaction of Hsc70 with CapZ, a protein that caps the barbed ends of Actin filaments that extend into Z-disk [230]. Loss of BAG3 makes CapZ more vulnerable to degradation resulting in loss of CapZ and fibre fragmentation following mechanical stress [230].



Mutations are coloured according to the disease classification. Dashed segments in cDNA represent the UTRs that are not drawn to scale.

Figure 6. Schematic representation BAG3 domains and mutations.

In summary, the most recently identified MFM causing gene plays key roles in the localisation of CapZ to the Z-disk through its interaction with Hsc70, protein folding and degradation, and in the regulation of apoptosis. Given the indirect role BAG3 plays in Z-disk and muscle function, its binding partners are excellent candidates for further genes that may be mutated in MFM. However, given the fact that most of the identified disease causing mutations lie outside a recognised domain in BAG3 there may be many more functions for BAG3 that remain to be characterised, perhaps including a direct role in the Z-disk.

8. Conclusion

The many functions of the MFM proteins, which are themselves just a very small subset of the Z-disk associated proteins, highlights the complex and dynamic nature of the Z-disk. Whilst a characteristic feature of MFM is dissolution of the myofibril, originating at the Z-disk, these disorders are not due to simple loss or disruption of structural components in

this tensile load bearing structure. This is exemplified by the identification of BAG3, which localises to the Z-disk but appears to only have indirect association to it, as an MFM protein. Further support comes from analysis of mouse knockouts for the MFM genes, none of which have defects in the formation of myofibrils.

The progressive nature of the disease and the identification of roles for the associated proteins in muscle repair and maintenance is more suggestive of a gradual accumulation of defects in Z-disk organisation eventually leading to structural failure. An interesting finding from mice lacking the long isoforms of ZASP is that they have increased levels of the MFM proteins Myotilin, CRYAB, and FLNC as well as the extracellular matrix proteins β1D Integrin and the sarcoglycans [189]. Up-regulation of Z-disk components is observed in α-Actinin-3 knockout mice in which FLNC, Myotilin, ZASP, and CRYAB are up-regulated [249]. Increased levels of FLNC are also detected in patients with LGMD or Duchenne muscular dystrophy [213]. This data, together with that previously presented, strongly supports the idea that the MFM proteins are up-regulated to protect the sarcomere and ECM from damage, whether that damage is caused by muscle activity, mutation of muscle proteins, or increases in oxidative, metabolic, and other forms of cell stress. Whether this is through a general stress response pathway that up-regulates the expression of Z-disk associated proteins or through a more specific pathway that selectively target proteins based on the nature of the stress remains to be determined.

For more than half of the cases of MFM the causative mutation is not known. As we have described there are many binding partners for the known MFM proteins, mutations in which may account for some of these cases. Additionally there is evidence from experiments with Desmin that mutations in other genes may act as modifiers of disease. Far more mutations have been identified in Desmin than in other MFM genes and it may be that modifiers will be identified in other subtypes of MFM as larger cohorts are analysed. Given the hypothesised role for the MFM proteins in stress response it is possible that any mutations that result in cellular stress may modify the presentation of MFM perhaps accounting for some of the differences in age of onset. Furthermore, differences in stress between cardiac and skeletal tissues may explain the differences in symptoms between these tissues, even in individuals with the same mutation. As the application of whole genome sequencing to mutation detection in myopathy becomes more widespread it may be possible to identify potential modifiers and investigate their role in MFM.

The existing literature on MFM and the MFM associated proteins has identified many exciting avenues for investigation. To investigate these areas further animal models, modelling specific MFM mutations, are required that would allow for better characterisation of pathology and the progression of disease together with a consistent genetic background to allow the analysis of potential genetic modifiers. The development of better tools to investigate the function of the MFM proteins, together with the identification of further MFM genes and modifiers, will allow us to improve our understanding of the many diverse

and complex roles of these Z-disk associated proteins and move closer to the development of effective therapies for these conditions.

9. Abbreviations

ACD domain: α-crystallin domain
AVB: atrioventricular block
BAG3: Bcl2-related athanogene 3
CM: cardiomyopathy
CRYAA: αA-Crystallin
CRYAB: αB-Crystallin
DCM: dilated cardiomyopathy
DRM: Desmin-related myopathy
ECM: extracellular matrix
FLNC: Filamin C
HCM: hypertrophic cardiomyopathy
IF: intermediate filament
Ig: immunoglobulin
LVNCC: left ventricular non-compaction cardiomyopathy
MA: muscle atrophy
MFM: myofibrillar myopathy
MW: muscle weakness
PGM1: Phosphoglucomutase 1
PKC: Protein kinase C
SBM: spheroid body myopathy
ULF: unit length filament
UPS: ubiquitin proteasome system
UTR: untranslated region
VEFR: ventricular ejection fraction reduction
ZASP: Z-band alternatively spliced PDZ-motif protein
WT: wildtype
ZM: ZASP/Cypher-like motif
GFAP: Glial fibrillary acidic protein

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Muscle Fibre Phenotyping from a Single Section: Is It as Informative as from Serial Sections?

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Additional information is available at the end of the chapter

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1. Introduction

Major sarcomeric thick filaments' protein of mammalian skeletal muscles is myosin. The thick myosin filament is composed of hexameric myosin molecules consisting of 2 myosin heavy chain (MyHC) subunits and 4 myosin light chain subunits (2 pairs of nonidentical light chains). The globular, amino terminal head domain, of MyHC has binding sites for actin and ATP. The elongated, alpha-helical coiled-coil carboxyl-terminal rod domain of MyHC exhibits filament forming properties, for review see (1). Several distinct MyHC isoforms exist. Human limb and trunk muscles contain muscle fibres expressing skeletal muscle-specific MyHC isoform IIA (MyHC-IIA) encoded by MYH2 gene on chromosome 17, MyHC isoform IID/IIX (MyHC-IIX) (2-4) encoded by MYH1 gene on chromosome 17 and an isoform that is expressed in both skeletal and cardiac muscle MyHC-β or MyHC-I encoded by MYH7 gene on chromosome 14 (5, 6). Two developmental myosin heavy chain isoforms (MyHC- embryonic and MyHC- perinatal) may be present in adult muscles, particularly in regenerating muscle fibres. MyHC family in humans includes also cardiac specific isoform MyHC-α and specialized isoform expressed in extraocular muscles (MyHC-extraocular) (6, 7). The forth skeletal muscle-specific isoform MyHC-IIB, is expressed in mice and rat skeletal muscles and is thought not to be expressed in adult human muscles at the protein level; however MyHC-IIB mRNA has been detected in specialized human muscles (8, 9) and in limb muscles of patients suffering from Duchenne muscular dystrophy (10), suggesting that MyHC-IIB gene in humans can be reactivated in skeletal muscles undergoing profound degeneration/regeneration.

In humans MyHC isoforms could be expressed either as single isoforms (in pure fibres-I, IIA and IIX) or co-expressed in different proportions in hybrid fibres (mainly -I/IIA, -IIA/IIX and -I/IIA/IIX). Hybrid fibres could reflect either a state of transition toward an isoform that is stimulus dependent or a state of stability (11). In normal conditions the proportion of hybrid

fibres depends on age, gender and physical activity: The studies of Williamson et al. (11, 12) showed that in young untrained women the proportion of hybrid fibres was 30%, in young untrained man the proportion was 40% (12) and in older untrained man the proportion was about 30% (11). In young and old the proportion of hybrid fibres decreased with progressive resistance training (11, 12) (though differences exist with respect to the type of MyHC upregulation).

Muscle fibre typing which includes subclassification of fast fibres (distinction between IIA and IIX fibres) and identification of hybrid fibres by the type of MyHC isoform or by the activity of myofibrillar ATP-ase is technically quite a demanding process, since it requires serial muscle sections, is time consuming and expensive. Besides, identification of hybrid fast fibres by ATP-ase activity is generally not accurate (11).

Identification of muscle fibre types on a single muscle section applying specific antibodies to MyHC isoforms could enhance and simplify the phenotyping (13, 14). Gregorevic et al. (13) presented on animal muscles sequential staining of a single section with three different MyHC specific antibodies (MyHC-I, MyHC-IIA, MyHC-IIB) labelled with different fluorophores. Raheem et al. (14) applied on human muscles sequential immunohistochemical staining of a muscle section with antibodies against two MyHC isoforms (MyHC-I and MyHC-IIA), where first antigen was visualized with peroxidase and the second with alkaline phosphatase-conjugated secondary antibodies. Neither Gregorevic et al. (13) nor Raheem et al. (14) identified IIX fibres with antibodies specific to MyHC-IIX, but only by exclusion, i.e. fibres not immunoreactive with antibodies to MyHC-I, MyHC-IIA and MyHC-IIB in animals or MyHC-I and MyHC-IIA in humans were identified as IIX fibres.

In this study we compare methods for muscle fibre phenotyping on a single section with those on serial sections and discuss the advantages and limits of muscle phenotyping on a single section. In addition we demonstrate that A4.74 antibody, otherwise declared to be specific for MyHC-IIA in humans (14, 15), cross-immunoreacts with IIX fibres in humans, consistent with Smerdu and Soukup (16).

2. Methods

Muscle fibre phenotyping was performed according to the expression of myosin heavy chain isoforms (MyHC) by indirect immunoperoxidase method using anti-mouse immunoglobulins as secondary antibodies (P260, DAKO) as described previously (4, 16), except for IIX fibres where Novolink polymer detection system (Novocastra secondary antibodies, Leica Microsystems) was used as recommended by the manufacturer. Briefly *slow* fibres were demonstrated by BA-D5 antibody immunoreactive with β/slow MyHC-I in rats (2) and humans (5) in a dilution 1:100; *fast* fibres were demonstrated by A4.74 antibody (former Alexis Biochemicals, now Enzo Life Sciences) immunoreactive according to the product data sheet in rats and humans with MyHC-IIA, but according to (16) with MyHC-IIA and MyHC-IIX in humans and in dogs (17). A dilution of A4.74 was 1:100. Immunoreaction using A4.74 antibody was developed either with peroxidase-conjugated

anti-mouse immunoglobulins (P260, DAKO) or with alkaline phosphatase-conjugated anti-mouse immunoglobulins (D486, DAKO). IIX fibres were demonstrated by 6H1 antibody developed by Lucas (18) in a dilution 1:3000. BF-35 antibody which stains all muscle fibres, except fibres expressing MyHC-IIX (2) was used undiluted. Muscle fibre phenotyping on serial muscle sections was compared with two double immunoenzyme staining methods (i) described by Raheem et al. (14) and (ii) our own modification. Briefly, in (i) a single muscle section was successively treated with antibodies against MyHC-I (BAD5) and antibodies declared as specific against MyHC–IIA (A4.74). First primary antibody was visualized with peroxidase and the second with alkaline phosphatase. BCIP/NBT (Sigma) was used as a substrate for alkaline phosphatase. In the second modification (ii) we successively applied antibodies against MyHC-IIX (6H1) and antibodies declared as specific against MyHC–IIA (A4.74). Autopsy samples of vastus lateralis muscle of two healthy males who died suddenly were obtained within 12 hours after death. Muscle sampling was approved by the National Medical Ethical Committee of the Republic of Slovenia.

3. Results

Consistent with (16) we demonstrated that antibody A4.74 reacts in human vastus lateralis muscle with both MyHC-IIA and MyHC-IIX fibres (Fig. 1 and Fig. 2).

Higher dilutions of A4.74 antibodies did not eliminate staining of IIX fibres at dilutions at which IIA fibres could be stained reasonably well (results not shown).

Comparison of fibre phenotyping with the method of Raheem et al. (14) and fibre phenotyping on serial sections is presented in Fig. 3.

Double immunoenzyme staining method (Fig. 3a) demonstrates well muscle fibres expressing MyHC-I (slow fibres), all fast fibres (IIA and IIX fibres) as one group, as well as hybrid fibres IIA/I in which MyHC-IIA predominates (IIA>I). However the relative proportion of individual MyHC isoforms can not be estimated from a single section. Hybrid fibres IIA/I with similar contributions of MyHC-IIA and MyHC-I (IIA~I) (upper fibre with white asterisk in Fig. 3) and hybrid fibres with higher proportion of MyHC-I as MyHC-IIA (I>IIA) (lower fibre with white asterisk in Fig. 3) can not be recognised on a single section but only on serial sections. Hybrid IIA/IIX fibres, expressing MyHC-IIA and MyHC-IIX (fibres labelled with black dots (•) in Fig. 3) can be identified on serial sections only, because with the double staining method (and with A4.74 antibodies – Fig. 3b) they can not be distinguished from IIA fibres.

Distinguishing between slow and fast fibres can be successfully accomplished already with a single type of antibodies as BAD5 antibodies (stain all slow fibres) or A4.74 antibodies (stain all fast fibres) in humans. Unstained fibres in either case belong to other main group of muscle fibres (fast – Fig. 3c or slow – Fig. 3b).

Subtyping of fast fibres is more difficult. Antibodies to both, MyHC-IIA and MyHC-IIX have to be used. To see, whether it is possible to determine hybrid IIX/IIA fibres on a single

section, we successively applied antibodies against MyHC-IIX (6H1) and antibodies against MyHC-IIA + MyHC-IIX (A4.74) and compared the results with subtyping of fast fibres on serial sections and with double immunoenzyme staining method of Raheem et al. (14) (Fig. 4).

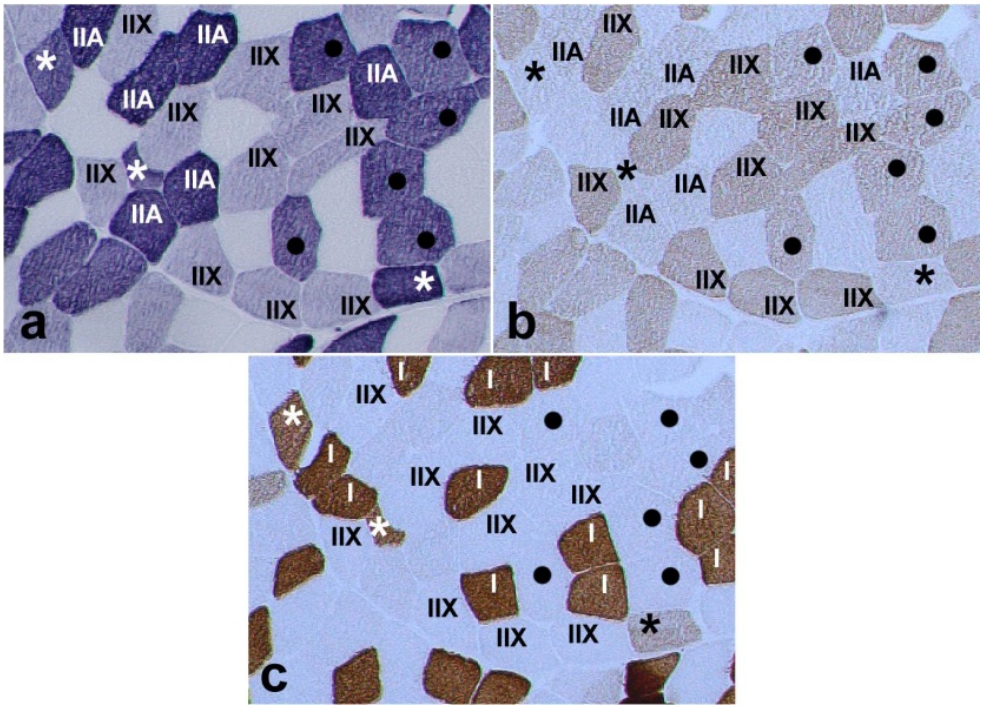

Figure 1. Immunoreactivity of A4.74 antibodies compared to immunoreactivity of 6H1 and BAD-5 antibodies on serial sections of human vastus lateralis muscle.
(a): A4.74 antibodies were declared as specific for MyHC-IIA, however A4.74 antibodies render an intense immunoreactivity in IIA fibres, in hybrid IIA/IIX fibres and a *moderate* immunoreactivity in *IIX* fibres. They do not stain slow fibres. (b): 6H1 antibodies specific for MyHC-IIX have relatively intense immunoreactivity in IIX fibres and hybrid IIA/IIX fibres. They do not stain IIA fibres and slow fibres. (c): BAD-5 antibodies specific for MyHC-I have intense immunoreactivity in I fibres. They do not stain IIA, IIX and hybrid IIA/IIX fibres.
Fibres labelled with dots (•): hybrid fibres IIA/IIX which express MyHC-IIA (a) and MyHC-IIX (b) and do *not* express MyHC-I (c). Fibres labelled with asterisks (*): hybrid fibres IIA/I which express MyHC-IIA (a) and MyHC-I (c) and do *not* express MyHC-IIX (b). IIA fibres: intense immunoreactivity with A4.74 antibodies, negative with 6H1 antibodies and BAD-5 antibodies. IIX fibres: relatively intense immunoreactivity with 6H1 antibodies (b), moderate immunoreactivity with A4.74 antibodies (a) and negative with BAD-5 antibodies (c). Slow or I fibres: intense immunoreactivity with BAD-5 antibodies (c), negative with A4.74 and 6H1 antibodies.

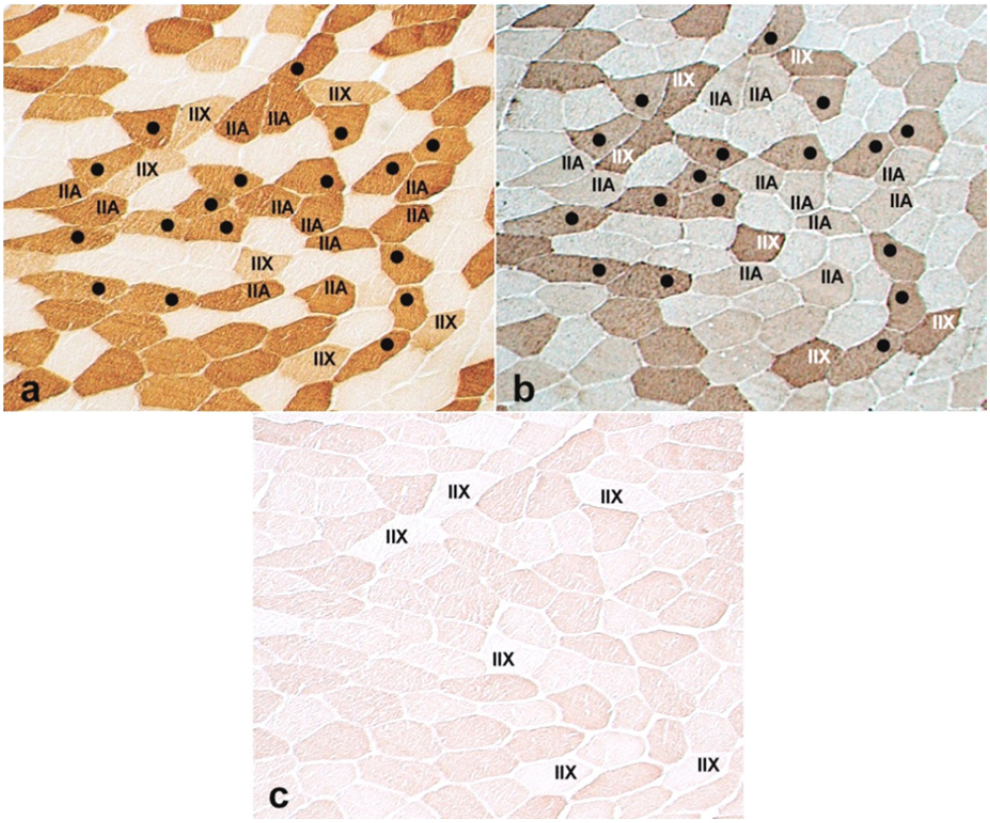

Figure 2. Immunoreactivity of A4.74 antibodies compared to immunoreactivity of 6H1 and BF-35 antibodies on serial sections of human vastus lateralis muscle.
(a): A4.74 antibodies show an intense immunoreactivity in IIA fibres and hybrid IIA/IIX fibres and also a *moderate immunoreactivity in IIX fibres*. (b): 6H1 antibodies have intense immunoreactivity in IIX fibres and hybrid IIA/IIX fibres. (c): BF-35 antibodies stain all muscle fibres except "pure" IIX fibres which do not co-express other MyHC isoforms. Fibres labelled with dots (•): hybrid IIA/IIX fibres which express MyHC-IIA (a) and MyHC-IIX (b) and are all immunoreactive with BF-35 antibody (c). IIX fibres: intense immunoreactivity with 6H1 antibodies (b), moderate immunoreactivity with A4.74 antibodies (a) and negative with BF-35 antibodies (c).

Due to cross-immunoreactivity of A4.74 antibodies with pure IIX fibres separation of IIX and other fast fibres (IIA fibres and IIA/IIX fibres as a group) is possible already with A4.74 antibodies where unstained fibres are slow fibres (Fig. 1a, Fig. 2a, Fig. 3b, Fig. 4b). A4.74 antibodies alone can not distinguish between "pure" IIA fibres and hybrid IIA/IIX fibres. With double immunostaining of IIA and IIX fibres (Fig. 4c) no additional information is provided. Hybrid fibres IIA/I can not be visualised with this method (fibres labelled with arrows and white asterisks in Fig. 4).

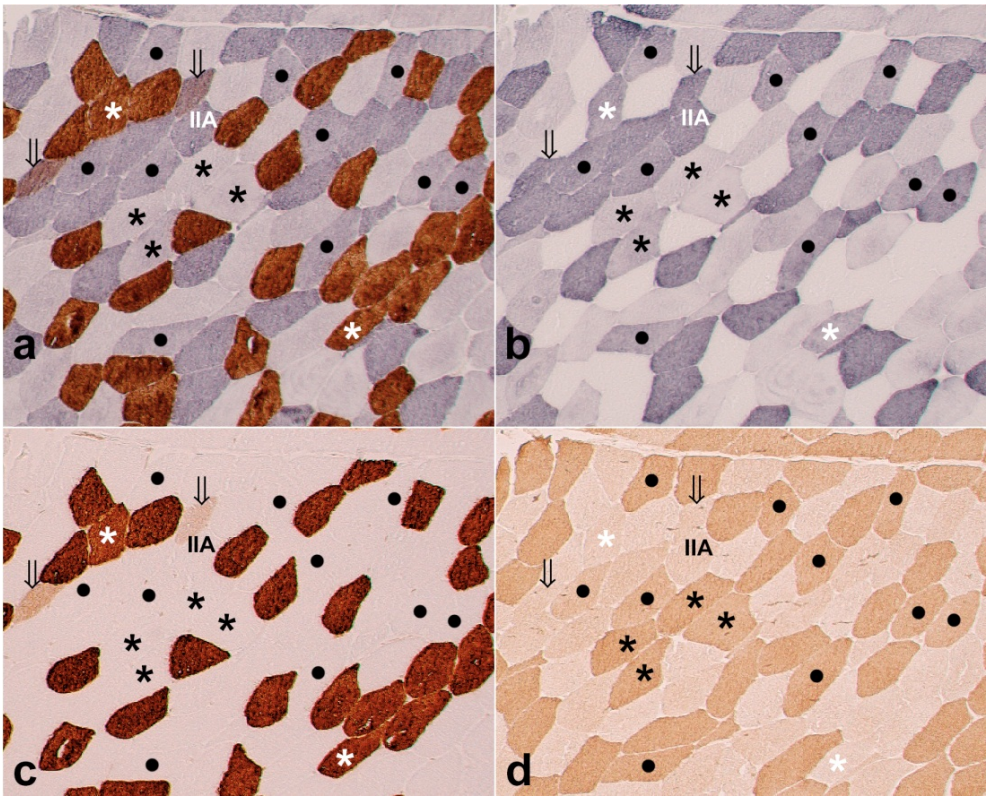

Figure 3. Phenotyping of muscle fibres with the double immunoenzyme staining method according to Raheem et al. (14) on a single section compared to phenotyping of muscle fibres on serial sections. (a): Double immunoenzyme staining – applying antibodies against MyHC-I (BAD5) and antibodies against MyHC-IIA + MyHC-IIX (A4.74). (b-d): Immunohistochemical reactions with individual antibodies. (b): against MyHC-IIA + MyHC-IIX (A4.74) visualized with alkaline phosphatase. (c): against MyHC-I (BAD-5) visualized with horseradish peroxidase. (d): against MyHC-IIX (6H1) visualized with horseradish peroxidase. Fibres labelled with *black* asterisks (*) are IIX fibres. Fibers labelled with black dots (•) are hybrid IIA/IIX fibres. Fibres labelled with arrows are hybrid IIA/I (IIA>I) fibres. Fibres labelled with *white* asterisks (*) are hybrid IIA/I (IIA~I) or (I>IIA) fibres (upper and lower fibre).

Neither double staining method identifies hybrid fibres co-expressing MyHC-IIA and MyHC-IIX (IIA/IIX). Hybrid fibres IIA/IIX can not be separated either form IIA fibres (in double immunostaining with BAD-5 and A4.74 antibody) ((Fig. 3a, Fig. 4a)) or from IIX fibres (in double immunostaining with 6H1 and A4.74 antibody) (Fig. 4c).

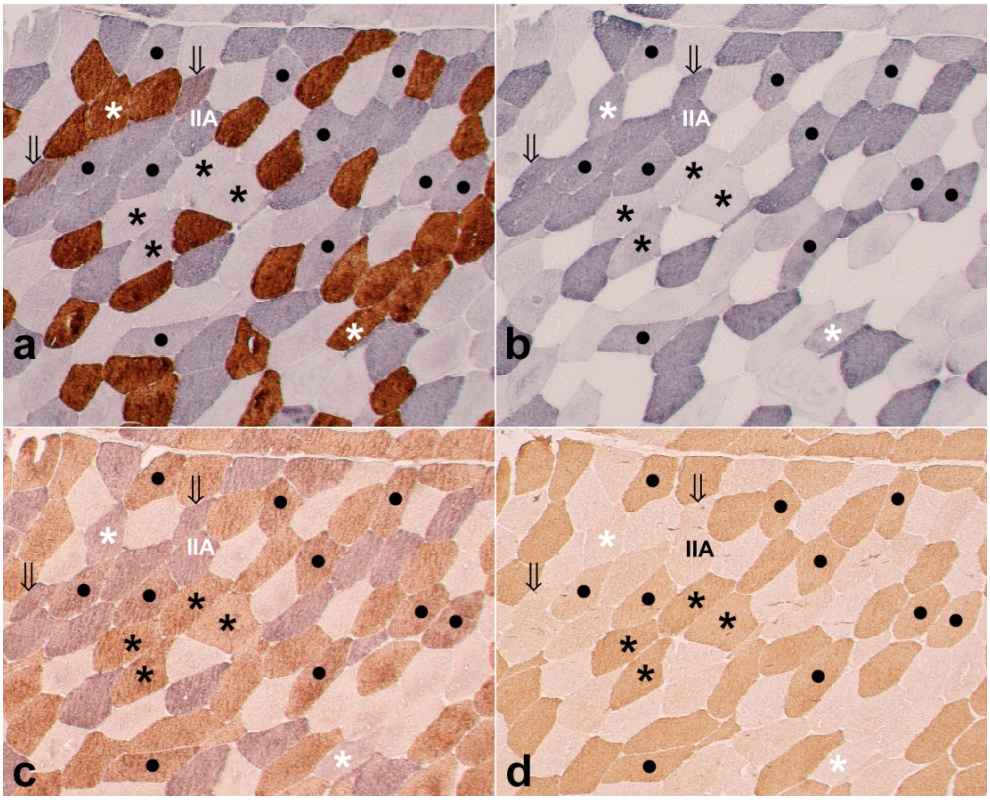

Figure 4. Double immunoenzyme staining according to Raheem et. al. (14) and double immunoenzyme staining of fast (IIA and IIX) fibres compared to phenotyping of muscle fibres on serial sections. (a): Double immunoenzyme staining method according to Raheem et al. (14) successively applying antibodies against MyHC-I (BAD5) and antibodies against MyHC-IIA+ MyHC-IIX (A4.74). (c): Double immunoenzyme staining of fast fibres successively applying antibodies against MyHC IIX (6H1) and antibodies against MyHC-IIA+ MyHC-IIX (A4.74). (b) and (d) are immunohistochemical reactions with individual antibodies. (b): against MyHC-IIA+ MyHC-IIX (A4.74) visualized with alkaline phosphatase. (d): against MyHC-IIX (6H1) visualized with horseradish peroxidase. Fibres labelled with *black* asterisks (*) are IIX fibres. Fibres labelled with black dots (•) are hybrid IIA/IIX fibres. Fibres labelled with arrows are hybrid IIA/I (IIA>I) fibres. Fibres labelled with *white* asterisks (*) are hybrid IIA/I (IIA~I) or (I>IIA) fibres (upper and lower fibre).

4. Discussion

Similarly to Smerdu and Soukup (16) we have demonstrated in human vastus lateralis muscle that A4.74 antibodies which should be specific for muscle fibres expressing MyHC-IIA weakly stain also MyHC-IIX expressing fibres. The latter were not hybrid IIX/IIA fibres, but pure IIX fibres, since the corresponding fibres were negative with BF-35 antibodies, which stain all muscle fibres, except fibres expressing MyHC-IIX (2, 19). Smerdu and

Soukup (16) showed that muscle fibres stained weakly with A4.74 antibodies and strongly with antibodies specific for MyHC-IIX (6H1) in humans expressed exclusively MyHC-IIX mRNA, which further confirms that weakly stained fibres are pure IIX fibres. Recently, Bloemberg and Quadrilatero (20) showed that SC-71 antibody, another antibody directed against MyHC-IIA, also cross-reacts with IIX fibres, as already also described by Smerdu and Soukup (16). A4.74 antibodies thus stain dark fibres expressing MyHC-IIA (fibres IIA and hybrid fibres IIA/IIX) and stain intermediate fibres expressing MyHC-IIX (Fig.1a, Fig. 2a, Fig. 3b, and Fig. 4b).

Both techniques for phenotyping of muscle fibres on a single section, triple immunofluorescent method (13) and double immunoenzyme staining method (14) did not directly identify IIX fibres (by antibodies to the MyHC-IIX). We have demonstrated that hybrid fibres IIX/IIA are missed by double immunoenzyme staining method (14) as they are misclassified as IIA fibres (Fig. 3a). Similar would hold also for the triple immunofluorescence method mentioned above, as antibodies to the MyHC-IIX were not used. The colour of the reaction product in hybrid IIX/IIA fibres, which are not recognised as such, does not change (remains blue) during superposition on immune reaction with antibodies directed against MyHC-I, since MyHC-I are not expressed in these hybrid fibres. We have clearly demonstrated (Fig. 3) that the staining intensity of MyHC-IIA in hybrid fibres can be as intensive as in pure IIA fibres, but to prove that such a fibre is a hybrid fibre, serial sections including immunohistochemical reaction to MyHC-IIX must be analysed.

The hybrid fibres IIA/I can be demonstrated with this method, if the proportion of MyHC-IIA predominates (IIA>I) in hybrid fibres (fibres labelled with arrows in Fig. 3), but if proportions are similar (IIA~I) or MyHC-1 predominates (I>IIA), such fibres are missed (fibres labelled with white asterisks in Fig. 3).

We conclude that hybrid fibres can be reliably demonstrated only on serial muscle sections. They may be demonstrated on a single section, if staining with antibodies directed to both MyHC expressed in hybrid fibres are used, but this may not be valid for all proportions of individual MyHC in hybrid fibres. By no means can hybrid fibres co-expressing three types of different MyHC be demonstrated by the double staining method. Nevertheless, the method of Raheem et al. (14) was not developed for demonstration of hybrid fibres, but as an alternative to routine ATP-ase staining in diagnostics of myopathies and can be particularly useful for demonstrating of advanced atrophic fibres in conditions as myotonic dystrophy type 2 (14) which justifies it as a good method for routine diagnostics.

The double immunoenzyme staining method (14) in addition presents well slow fibres expressing MyHC-I and all fast fibres, expressing either MyHC-IIA or MyHC-IIX, but IIA and hybrid IIA/IIX fibres can not be distinguished. The distinction between fast and slow fibres can be accomplished already by staining of muscle section with antibodies to MyHC-I alone. Double immunoenzyme staining offers more clear presentation of fast fibres' contours, which may be as “unstained” fibres difficult to present in sections stained only with antibodies to MyHC-I (Fig. 3c), especially in clusters of fast fibres. Alternatively, antibodies to MyHC-IIA, as A4.74 and SC-71, which also cross-react with MyHC-IIX (16, 20)

can also be used to separate fast and slow fibres on a single section. In this case slow fibres are unstained (Fig. 3b or Fig. 4b). Obviously the advantage of the double immunoenzyme staining of Raheem et al. (14) is in (i) the clear simultaneous presentation of fast and slow fibres and (ii) presentation of "pure" IIX fibres (not co-expressing other MyHC isoforms); the latter is possible due to cross-reactivity of antibodies to fast fibres (A4.74 and SC-71) as mentioned above, but identification of hybrid fibres is unsatisfactory and therefore also the separation of IIA fibres and hybrid fibres co-expressing MyHC-IIA and MyHC-IIX.

In our own modification of double immunoenzyme staining method in which we superimposed two immunohistochemical reactions for demonstration of fast fibres (IIA and IIX), we demonstrated that IIX fibres can not be reliably distinguished from hybrid fibres IIX/IIA, since the differences in colours were negligible (Fig. 4c). We consider this as an additional proof that demonstration of hybrid fibres on a single section is tricky, even if two corresponding antibodies to MyHC isoforms expected to be co-expressed in hybrid fibres are used. The mixed colour of the two reaction products may simply reflect the dominance of one colour and may not be correlated to the proportions of MyHC isoforms. In Fig. 4c in hybrid IIX/IIA fibres the brownish colour of MyHC-IIX isoforms completely covered the blue colour of the MyHC-IIA isoforms. Similar is valid also for the demonstration of hybrid fibres I/IIA (I~IIA and I>IIA), labelled with white asterisks in Fig. 3, in which strong brown colour of the reaction product of MyHC-I also completely covered the blue colour of MyHC-IIA. In addition, with double immunoenzyme staining technique it is assumed, that first primary antibody (in our case BAD-5) is completely coupled with the secondary antibodies (in our case peroxidase-conjugated secondary antibodies). However, the cross-immunoreactivity of secondary phosphatase-conjugated antibodies with the primary antibodies (BAD-5) can not be excluded. As expected we could not identify hybrid IIA/I fibres by superimposing two immunohistochemical reactions to fast fibres, as antibodies to MyHC-I were not used.

Recently Bloemberg and Quadrilatero (20) reported on rapid determination of myosin heavy chain expression on a single section using multicolour immunofluorescent method on animal and human muscle. In human muscle they used three different primary antibodies against different MyHC isoforms, including antibody to MyHC-IIX (18). The method takes the advantage of the fact that antibodies against MyHC isoforms, belong to different subtypes of immunoglobulins: IgG2b (against MyHC-I), IgG1 (against MyHC-IIA) and IgM (against MyHC-IIX). Muscle fibres immunoreactive with particular antibody can be visualised with secondary antibodies specific for only one subclass of immunoglobulins and labelled with different fluorophores. The authors were able to demonstrate major fibre types and hybrid I/IIA and IIX/IIA fibres. However, the identification of hybrid IIX/IIA fibres on merged images is not straightforward, but hybrid fibres can be recognised, if analysing all single channel images (see their Fig. 5). Also the reported percentage of hybrid fibres in this study (20) is much lower than in the previous studies using single fibre electrophoresis (11, 12). It seems that the advantage of multicolour fluorescent method is the possibility to analyse single channel images, that is similar to analysis of individual serial sections, to

identify especially hybrid fibres with two MyHC isoforms (20) and possible also with three MyHC isoforms, if in doubts on merged images.

If different fluorophores are directly coupled to primary antibodies more than three labelled antibodies can be used on the same section (13) and antibodies as MyHC-neonatal and MyHC-embryonic, which are expressed during development and regeneration, can be demonstrated on the same section with other myosin antibodies. In addition when using multicolour immunofluorescent method correlation between metabolic profile of muscle fibres and contractile characteristics can be studied more easily (20). Obviously metabolic profile can be identified in next two consecutive muscle sections where tracing of muscle fibres is easier than in more distant muscle sections. Adjustments of orientations of muscle profiles in distant sections are sometimes necessary with specialised computer programs for image analysis as (21). Theoretically total of six emission/detection channels can be used (13), if primary antibodies are directly coupled with fluorophores which have very narrow emission spectrum. Besides different antibodies to MyHC isoforms antibodies to other proteins labelled with distinct fluorophores may be included and the results correlated to contractile profiles of muscle fibres. However, when analysis is based on mixed colours full attention must be paid to possible misinterpretation of staining artefacts as hybrid fibres.

5. Conclusion

A good agreement was found between muscle fibre phenotyping on serial muscle sections and muscle fibre phenotyping on a single section by double immunohistochemical staining method, if muscle fibres expressed a single MyHC isoform. The identification of hybrid fibres expressing two different MyHC isoforms requires the superposition of both antibodies directed against MyHC isoforms which are expressed in hybrid fibres. Even then, the identification of hybrid fibres may not be successful: it depends on relative proportions of individual MyHC isoforms. In addition cross reactivity of secondary antibodies with primary antibodies may hinder identification of hybrid fibres. Hybrid fibres expressing three different MyHC isoforms can not be presented with the double immunohistochemical staining method.

Muscle fibre phenotyping on serial muscle sections was definitely superior to the double staining method performed on a single section, when *hybrid* fibres were present.

The multicolour immunofluorescent method applying three different antibodies to major adult MyHC isoforms in human skeletal muscle (MyHC-I, MyHC-IIA, and MyHC-IIX) on a single muscle section may be a promising tool in simplifying muscle phenotyping and in identifying hybrid fibres. Primary antibodies to MyHC isoforms directly labelled with fluorophores enable simultaneous use of larger number of antibodies against MyHC as mentioned above. Presentations of MyHC isoforms and other proteins on the same section would also be an interesting application of the multicolour fluorescent method in further studies. Nevertheless, combination of more reactions on a single section means also combination of all artefacts that accompany individual procedures. Therefore all combined

reaction procedures must be applied with full precaution; otherwise they might turn into misinterpretations of artefacts.

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