Chapter 9

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

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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-$\alpha$) Akt2 (PKB-$\beta$) and Akt3 (PKB$\gamma$). 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 GSK3$\beta$ (glycogen synthase kinase-3$\beta$) 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).

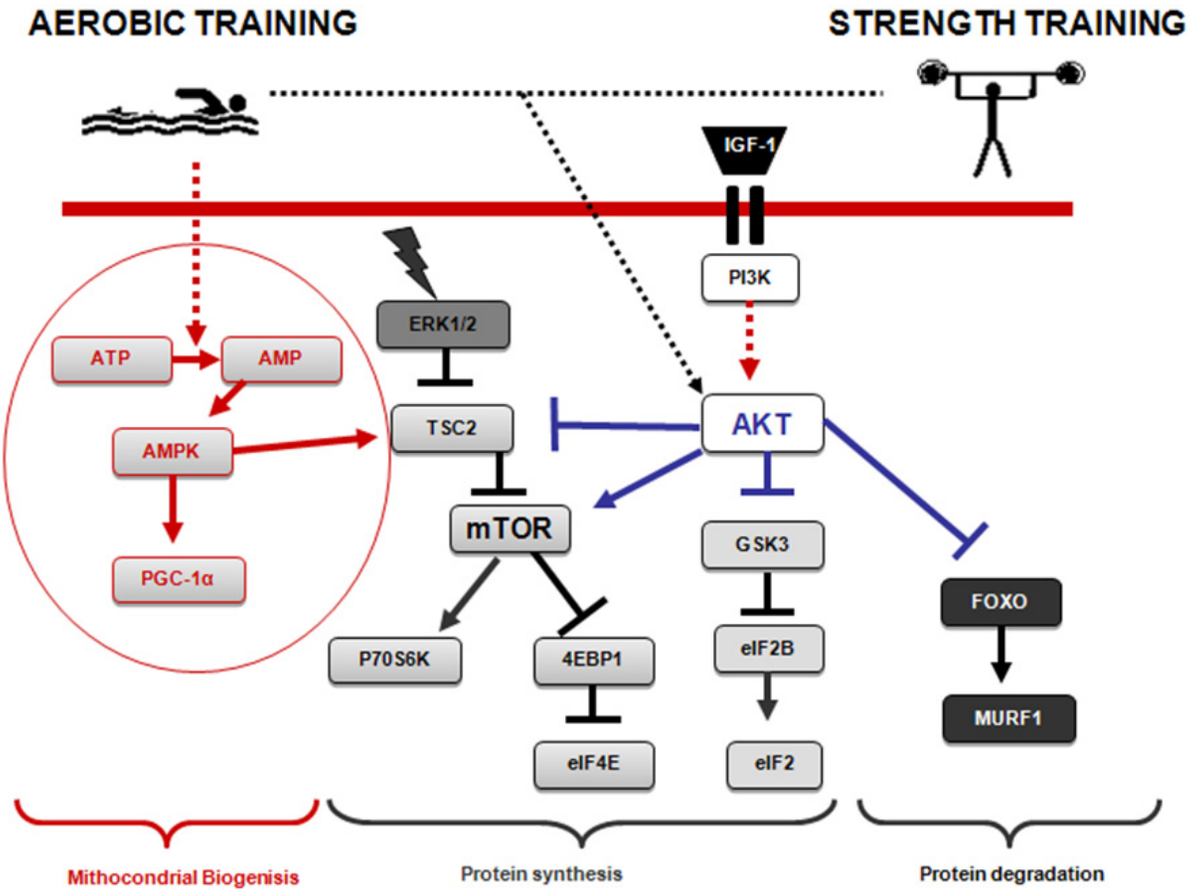


**Figure 1.** Signalling pathways regulated by exercise and/or IGF-I. Exercise has been shown to activate Akt and ERK1/2 directly, and also induces IGF-I synthesis in muscle. PI3K/Akt is of particular importance as it increases protein synthesis via mTOR activation and inhibits protein degradation via the inactivation of FOXO transcription factors with resistance training. AMPK-activation leads to PGC-1$\alpha$ 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-1$\alpha$= Peroxisome proliferator-activated receptor-$\gamma$ coactivator 1$\alpha$.

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-1$\alpha$, 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-1$\alpha$ 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).

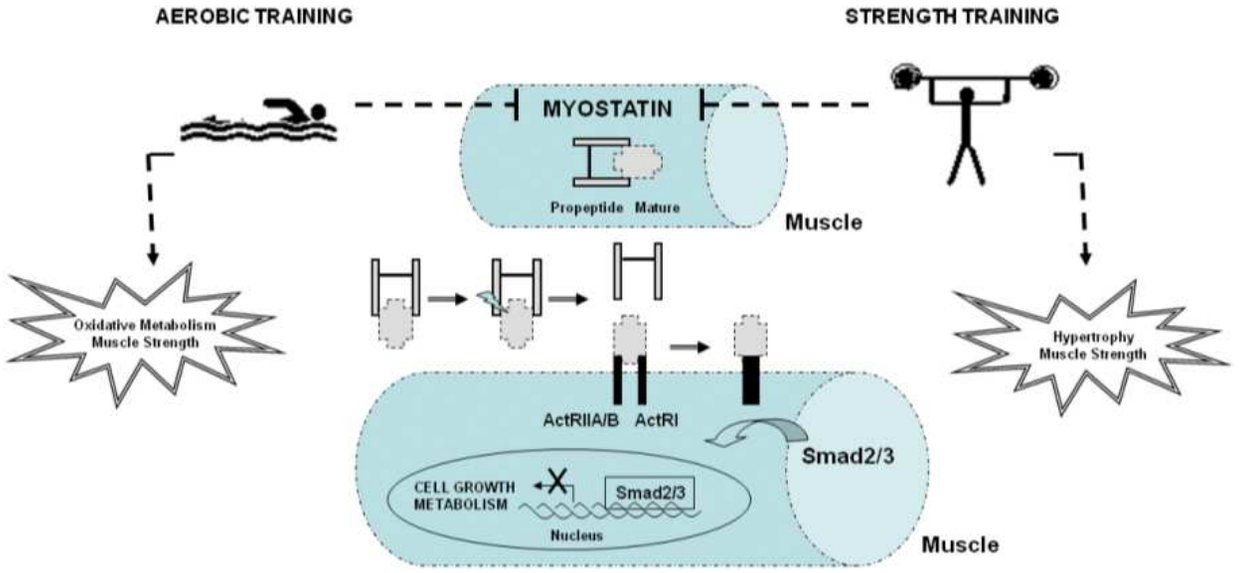


**Figure 2.** Effect of exercise training on skeletal muscle myostatin expression. Inactive myostatin (propeptide portion forms non-covalent link to mature portion) is secreted by muscle cells. Proteases on muscle cells release propeptide from mature region. Mature region binds type II Activin receptor. 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).

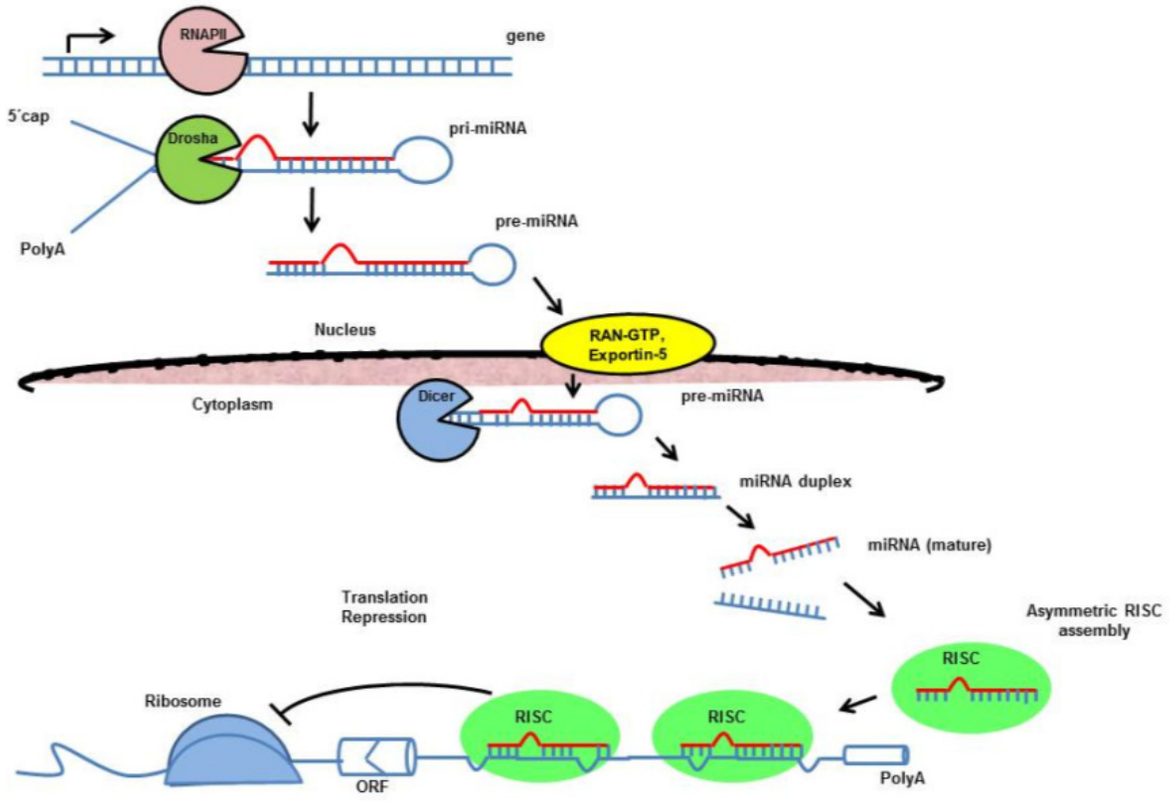


**Figure 3.** The Current model for the biogenesis and post-transcriptional supresssion of microRNAs – MicroRNAs are generally transcribed by RNA polymerase II (RNAPII) to yield primary miRNAs (pri-miRNA) transcripts are first processed into ~70 nucleotide pre-miRNAs by Drosha inside nucleus. Pre-miRNAs 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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