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2 Molecular mechanisms of skeletal muscle hypertrophy
Using molecular biology to understand muscle growth

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Abstract

Skeletal muscle has a tremendous capacity to adapt to the functional and metabolic loads placed upon it. This plasticity is most apparent in the physique of the strength athlete who has utilized the principles of progressive resistance exercise to improve their skeletal muscle mass and function. The most striking adaptation to progressive resistance exercise is muscle hypertrophy: the postnatal growth of muscle fibres. Since the pioneering work of Goldberg in the 1960s, a great deal of research has been carried out to understand the mechanisms governing this adaptation. Two cellular responses are

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required for hypertrophy: increased protein synthesis and an altered transcriptional profile. The control of protein synthesis is regulated by the mammalian target of rapamycin complex 1 (mTORC1), while the changes in mRNA levels could be due to β-catenin and/or micro-RNAs. mTORC1 is a kinase that controls the rate and capacity for translation, β-catenin is a regulator of transcription, and microRNAs act post-transcriptionally to suppress expression of families of mRNA that control muscle size, cellular differentiation and growth. The aim of this chapter is to discuss the regulation of these and other key molecules by resistance exercise and to highlight their respective roles in skeletal muscle hypertrophy. A secondary aim is to distinguish between muscle growth per se and resistance exercise-induced muscle hypertrophy highlighting the fact that a molecule may be involved in postnatal muscle growth without affecting hypertrophy.

1. Introduction

The term "Progressive Resistance Exercise" was first developed by two physicians, DeLorme and Watkins, shortly after World War II [1]. The term was used to describe a method of exercise that improved the muscle function of recovering Polio patients. The patients started off lifting a manageable weight. During the training period, the weight was increased progressively so that after some time their manageable weight was heavier than at the beginning. This example of training follows the overload principle – the stimulus is of sufficient frequency, intensity and duration to illicit an adaptation. The adaptation in this example is improved muscle strength and mass. The overload principle in the form of progressive resistance exercise has been exploited by athletes for millennia as a means to increase their muscle mass and strength to gain a competitive edge. Recently however, other populations have become interested in increasing muscle mass and strength. Due to improved longevity [2], health care systems are facing a burden in the care of frail elderly [3,4]. Furthermore, a number of disease states result in frailty including: HIV/AIDS [5], Cancer [6], Sepsis [7], COPD [8], and Diabetes [9]. This frailty is largely due to the loss of muscle mass [10,11]. Treatments that prevent muscle wasting or stimulate muscle growth would relieve much of this burden and improve the quality of countless lives. For this reason the molecular mechanisms of resistance exercise-induced skeletal muscle hypertrophy have been intensively studied for the past 40 years.

1.1 Models of resistance exercise and hypertrophy

Human resistance exercise

A typical program of human resistance training results in an average muscle enlargement of 0.1% per day. These programs typically consist of 1-5

sets of 6-10 different exercises that target specific muscle groups. Each set consists of 6-8 repetitions with loads between 67 and 75% of maximal voluntary force. A typical one set program will take 20 minutes to complete, while a 3 set program will take approximately 1 hour. During this time, muscle is performing active work for between 8 and 24 minutes and this is done 2-3 days a week [12]. This means that work-induced muscle hypertrophy results from as little as 20 minutes of high intensity muscle contraction a week.

A single bout of this type of resistance exercise in the fasted state can increase both the rate of protein degradation [13,14] and synthesis for 36-48hrs [13-16]. With proper nutrition, synthesis can be further increased while degradation is decreased resulting in a net increase in protein balance [17-20]. After repeated bouts of resistance exercise, the intermittent changes in protein turnover accumulate resulting in a new steady state with a greater number of myofibrils in parallel [21], increasing the radiological density [22] and radial diameter [21-23] of the muscle and improving the capacity to produce force [21-23]. However, studying molecular responses to exercise in humans is limited by the variability in responsiveness to exercise [24], the small amounts of tissue that can be collected in such studies, and difficulties in recruiting and maintaining subjects on a study for a prolonged period. As skeletal muscle hypertrophy is evolutionarily conserved across many species, several animal and tissue culture models have been developed to aide in the study of hypertrophy.

Animal studies

Since the seminal work of Goldberg in the 1960's [25], models of human resistance exercise have been developed for animals as diverse as quails, mice, rats, cats and horses. These models have been extremely useful for describing the mechanisms underlying muscle hypertrophy. Animal models have five primary advantages over human studies: (1) inbred strains of animals reduce genetic variation; (2) nutrition and environmental factors can be tightly regulated; (3) drugs and radioactive tracers can be used; (4) whole muscles can be removed for a variety of functional, biochemical, histochemical, and genetic analyses; and most importantly, (5) genetic engineering can be utilised to assess the contribution of specific genes to the hypertrophic process. There is little doubt that the introduction of animal models to study hypertrophy has greatly advanced our molecular understanding of skeletal muscle growth. However, the animal data must be interpreted with caution; keeping the physiological relevance of the model in mind. When considering the physiological relevance of an animal model we must take into account several questions: (1) how closely does the intervention replicate human progressive resistance exercise? (2) what is the time course of the growth and is this

comparable to the time course in humans relative to the life span? and (3) are the biochemical adaptations within the muscle comparable to human progressive resistance exercise? When using these criteria, animal models can be largely separated into two categories: those that are physiological and those that are non-physiological. We will give only a brief description of the animal models that are currently used to study the molecular control of muscle hypertrophy and compare these with human muscle hypertrophy. For more in depth analysis of this area see reviews by Booth & Thomason [12], Timson [26], and Lowe & Alway [27].

Non-physiological models of hypertrophy

The first rodent muscle hypertrophy models developed in the 1940's were based on the finding that passive stretch is sufficient to induce growth. For instance, immobilising the ankle joint with the soleus in a stretched position [28] or denervating one half of the diaphragm [29] resulted in hypertrophy that was followed by atrophy of the immobilised/denervated muscle. As these models were ineffective at maintaining hypertrophy, other stretch models have since replaced them. Avian stretch models, whereby weights are attached to the wing of an adult chicken have been used to induce an 80% hypertrophy (measured by wet weight) in the first week followed by a further doubling of muscle size by 5 weeks in the anterior latissimus dorsi (ALD) muscle [30]. This model has been widely used to study the genetic response to a hypertrophic stimulus [31]. Although these models are effective at inducing and maintaining hypertrophy, they do not replicate human hypertrophy, the time-course is much faster, and some of the phenotypic changes, specifically the hyperplastic response, are unlike work-induced skeletal muscle hypertrophy.

A modified stretch model that also creates a load-induced hypertrophy is compensatory overload. This model involves severing the tendon (tenotomy), nerve (synergist denervation) or completely removing the synergist (synergist ablation) in a group of muscles, most commonly the gastrocnemius in rodents. The remaining muscles compensate for the loss of the synergist(s) by increasing their functional capacity. Tenotomy was developed in the 1960's and induces a rapid increase in the wet weight of the soleus and plantaris of 30-50% and 20% respectively within 6 days [32]. However, simple tenotomy permits the reattachment of the severed tendon increasing the variability of the model. To overcome this shortcoming, in the 1970's the synergist ablation model was developed. Removal of the tibialis anterior results in 15% hypertrophy of the remaining extensor digitorim longus within 4 days [33] and removal of the gastrocnemius results in a 103% and 45% increase in fibre area of the plantaris and soleus respectively after 60 days [34]. Due to the relative ease of the surgery and low contact time with the animals, synergist

ablation is the most widely used long-term model of skeletal muscle hypertrophy. However, the load is present whenever the animal is active (up to 12 hours a day) and this does not replicate human resistance exercise where the load is present for a few minutes a week. Typically, programs of human resistance exercise result in increased muscle volume, as measured by MRI, of 9.1-14% over periods of 9-14 weeks [35]. Compare this to the massive increases in mass seen over a matter of days with overload and clearly the overload models provide a much greater hypertrophic response than is possible in human work-induced hypertrophy. Lastly, compensatory hypertrophy models result in a shift in myosin isoform towards slow twitch fibres [36], whereas long-term resistance training in humans does not result in major changes in fibre type composition [37,38], even though a shift from Type IIB to Type IIA can be seen [39,40]. Therefore, even though overload is effective and broadly used, it is not a physiological model of muscle hypertrophy. For this reason, data from this model must be interpreted with a degree of caution.

Physiological models of skeletal muscle hypertrophy

As a result of concerns with the synergist ablation model, several groups have developed animal models of progressive resistance exercise that more closely mimic the loading patterns, growth response and biochemical adaptations seen in humans. Animal models designed to mimic human resistance exercise include operant conditioning and in vivo electrical stimulation. The first operant conditioning models were developed by Goneya & Ericson in 1976 [41]. These authors conditioned adult cats to flex their right paw against a footplate for a food reward. Once conditioned, the animals were exercised 5 days a week for up to 41 weeks and experienced an increase in wet weight of the flexors of 7-34%. The successful feline model was followed by several rodent variations in the 1980's, whereby rats were conditioned to perform squat type exercises for a food reward [42]. One such model developed by Ho et al. [43] involved conditioning rats to respond to a light stimulus and reach for a steel bar with weights attached to their abdomen, motivation came in the form of an electric shock. The rats were exercised for 4 days a week for 8 weeks. The program consisted of performing 16 squat like movements that took 2 secs to complete with a 30 sec rest between each squat. The weight was progressively increased until the rats were lifting 130% of their body weight resulting in a 12% increase in the mass of the adductor longus. These models are effective at inducing a degree of hypertrophy over a time course that more closely resembles the human response. However, they are very demanding on the researchers time and lack an inter-animal control.

The next generation of animal models came with the development of electrical stimulation in anaesthetised animals. Wong & Booth [44] developed

the first of these models in 1988 when they anaesthetised animals and used platinum electrodes to stimulate the muscles of the right leg. A footplate connected to a weighted pulley was strapped to the foot and upon stimulation the foot plantar flexed lifting a weight that was progressively increased over the training period. Various paradigms were utilized, with the paradigm of 4 sets of 6 repetitions with 3 days of recovery resulting in a doubling of the weight lifted and a 13-18% hypertrophy of the plantar flexors over 16 weeks of training. There was however, some concern over possible damage caused by applying an electrical current directly to the muscle. Consequently, Wong and Booth's model was modified so that the platinum wire electrodes were implanted on the right sciatic nerve instead. As all the muscles below the point of stimulation contract maximally, the foot plantar flexes due to the greater mass in the posterior compartment of the leg. As a result the muscles in the anterior compartment (tibialis anterior and extensor digitorim longus) contract eccentrically. The protocol consisted of 10 sets of 6 repetitions twice a week for 6 weeks. The end result was approximately 14% hypertrophy of the TA and EDL [80]. While both the electrical stimulation and operant conditioning models are very effective, the stimulation models require much less time and provide a greater degree of control over the exercise paradigm. In electrical stimulation, the investigator controls the number of repetitions and the rest periods during the program and the completion of the exercise bout is distinct so that effective measures of the dynamics after exercise can be made.

In vitro models

In order to gain even greater levels of control over experimental conditions, researchers have turned to ex vivo models of resistance exercise and tissue culture. Ex vivo whole muscle models were first used to study the response to hypertrophic stimuli in the 1970's (reviewed by Vandenberg, [45]). They presented several advantages over whole animal systems. The primary advantage was the control over the external milieu. The researcher could add or remove anything to the media and assess the effect on the exercise response. Also, a variety of stimulation patterns could be rapidly assessed using automatic servo motors set up to carry out various protocols. The major disadvantage of these models has been the inability to preserve whole muscles in culture for long periods due to difficulties in maintaining the nitrogen balance and cell death due to hypoxia [45]. Thus, ex vivo muscle models are only useful for studying acute effects and there are questions regarding whether the stretch or the hypoxic conditions lead to the changes measured.

Due to the problems associated with whole muscle ex vivo models, researchers have turned to tissue culture models. Cell or tissue culture techniques have been around since the late 1800's, however it took almost a further 150

years before tissue culture became widely used. Interestingly, like the development of progressive resistance exercise, the wide-scale use of tissue culture can be traced to Polio. In 1949, the discovery that cultures of animal cells could be used to generate the Poliovirus resulted in the development of the techniques required to generate large quantities of Poliovirus for vaccine production. Over time, a greater variety of cells were cultured with immortalised muscle cells produced in 1977 [46]. Vandenberg & Kauffman, [47] developed the first in vitro tissue culture model of skeletal muscle hypertrophy. They grew chicken embryonic myoblasts on a stretching frame until they differentiated into myotubes and then applied a stretch of 5-20% for 18 hours. These stretch protocols resulted in a 6-9.5% increase in total protein content. However, like the ex vivo muscle explant models, tissue culture systems lack systemic interactions. But a further limitation to tissue culture is that most of these experiments are preformed in 2-dimensions making it very difficult to measure the true functional changes that occurs within muscle. This particular disadvantage is gradually being overcome through the development of tissue engineering techniques that can consistently produce homogenous 3-dimensional muscle in culture, but these models are not widely in use at this time.

Obviously, the gold standard model for studying the molecular mechanism of skeletal muscle hypertrophy is human studies. However, due to the limitations of such studies other models are required. No single model is appropriate for all situations. However, it is important to use a range of different models and be cautious when interpreting data from non-physiological models of muscle hypertrophy. When determining the requirement of a specific gene for the molecular response to resistance exercise, overload in transgenic animals has been used exclusively. While this can give some general insight, it is then necessary to go back and use a more physiological model to determine what stage of the growth response is affected by the gene of interest. It is only in this way that we will completely understand how increased load is transduced to a signal that increases protein synthesis and alters gene expression.

1.2 Signalling to growth

The first major question in the field of skeletal muscle hypertrophy is; what is it about resistance exercise that is transduced into growth? Although there is no definitive answer to this question at the moment, there are clues as to what the muscle is sensing. First, stretch of the soleus by immobilising the ankle joint in a dorsi flexed position [28]; weight [30], or spring loaded devices [48] applied to chicken wings; and stretch in cultured muscle cells all induce hypertrophy [47]. Second, denervating the stretched muscle in the rat [49] or the chicken [30] does not prevent hypertrophy, whereas denervation

of the antagonistic muscles, that provide the stretch, does prevent growth [33]. Third, hypertrophy only occurs when sufficient weight is applied to the muscle [50,51]. Finally, fourth, hypertrophy can occur in the absence of external nutrients, pituitary or thyroid hormones or insulin [25]. Together, these early animal studies suggest that tension, either passive or active force across a muscle, is the signal that leads to muscle hypertrophy and points to a force sensor (mechanosensor) within the muscle that can transduce tension into a growth signal. However, even though some of the downstream signals have been identified, the identity of this force sensor has been allusive.

Integrin-associated signalling

The ideal position for a mechanosensor is within the region connecting the internal architecture of the cell to the extracellular matrix or to other cells [52]. A number of protein complexes serve this purpose in muscle including the dystrophin-associated glycoprotein complex, integrin associated costameric complex, and cell-cell adherins junctions. The role of some of these protein complexes in mechanosensing has been studied extensively in the heart. In the heart, proteins that are associated with costameres appear to be important in sensing mechanical stretch and initiating the hypertrophic response. Specifically, β-integrins [53,54], the integrin-linked kinase (ILK) [55], melusin [56,57], the muscle LIM domain protein [58], and protein kinase B/akt all are found in costameres and all play an important role in mechanosensing in the heart. Of specific interest are ILK and PKB/akt. ILK is both a kinase and a scaffolding protein. It interacts with β-integrins, PKB/akt, and the rapamycin-insensitive companion of mTOR (rictor). As will be discussed later, rictor activates PKB/akt and the activation of PKB/akt can lead to muscle hypertrophy in both the heart and skeletal muscle [59]. This suggests that ILK may coordinate the conversion of the mechanical stimulus from the β-integrins into the chemical signal of PKB phosphorylation.

Another member of costameres and adherins junctions is focal adhesion kinase (FAK) [60]. Work by Fluck et al. [61] demonstrated an increase in the amount and activity of FAK within 24-36 hours of the onset of stretch in the chicken ALD and in the rat soleus after overload. Furthermore, the degree of FAK expression and activity in muscles is related to their loading [62]. For instance, the postural soleus muscle has greater expression and tyrosine phosphorylation of FAK than the gastrocnemius or plantaris [62]. Moreover, unloading reduces the phosphorylation of FAK in the soleus and the concentration of FAK in the gastrocnemius and plantaris [62]. This suggests that load may be sensed through FAK. However, unlike ILK, it is unclear what the downstream targets of FAK may be involved in this process.

Calcium signalling

Calcium has also been proposed as a mechanosensor mediating hypertrophic responses. Guharay and Sachs [63] first identified stretch-activated ion channels in skeletal muscle in 1984. These channels respond to membrane deformation with increased open time allowing calcium influx that can be blocked by the antibiotic streptomycin and the cation gadolinium. Treating animals with either streptomycin or gadolinium prior to a bout of resistance exercise can decrease the signalling associated with muscle growth [64], suggesting that these channels may be important in mechanosensing. Further support for this theory comes from the fact that increasing intracellular calcium in muscle cells using the calcium ionophore A23187 enhances both protein synthesis and degradation rates [65] much like resistance exercise, and the calcineurin inhibitors, cyclosporin A and FK506, prevent growth factor-induced muscle hypertrophy in culture [66]. However, calcineurin is not activated by overload hypertrophy, pharmacological blockade with cyclosporin A does not prevent overload-induced muscle growth [59], and mice lacking calcineurin can still undergo overload-induced hypertrophy [67]. Futhermore, stretch in combination with A23187 enhanced protein synthesis further than treatment with A23187 alone [65]. Together, these data suggest that although calcium influx may play a role in mechanosensing, it is not the only sensor and it does not signal though calcineurin.

Growth factors

Growth factors released in an autocrine manner in response to stretch have also been proposed as a mechanosensor. Growth factors such as the IGFs (insulin like growth factors) have important roles in myogenesis and muscle growth through their ability to regulate proliferation, differentiation and growth hypertrophy [68]. Turner et al. [69] demonstrated that implanting growth hormone secreting GH3 cells into rats induced an increase in the expression of IGF-I (8-fold) and IGF-II (6-fold) and resulted in skeletal muscle hypertrophy. DeVol et al. [66] found that tenotomy of the gastrocnemius increased the mRNA expression of IGF-I and IGF-II in normal and hyposectamized animals, demonstrating that load could increase the expression of the IGFs independent of growth hormone. Stretch in rabbits also induces the expression of a splice variant of IGF-1 called mechano-growth factor (MGF) that may be invovled in muscle hypertrophy [70]. The role of growth factors in the response to hypertrophic stimuli has recently been brought into question by experiments using genetically modified mice overexpressing a dominant negative form of the IGF receptor [71]. These mice underwent normal overload hypertrophy despite being growth factor resistant, suggesting that while IGF-1 plays an important role in developmental

growth [71,72] IGFs may not play a role in resistance exercise-induced hypertrophy.

Other growth factors including fibroblast growth factor-2 (FGF2) are also released in response to mechanical stimulation. Using cultured human myotubes grown on stretching apparatus, Clarke and Feeback, [73] demonstrated that passive stretch induces the release of FGF2 into the culture media in a stretch dependant manner. Furthermore, antibodies directed against FGF2 inhibited the stretch-induced growth of the myotubes [73]. More recently Baar et al. [74], showed that conditioned media from stretched myotubes is sufficient to activate growth signalling when applied to unstretched myotubes. However, this was not confirmed in muscles mechanically stimulated ex vivo [88] suggesting that the data from Baar et al. [74] may be an artifact of 2D culture. Taken together, it is unlikely that autocrine/paracrine release of growth factors serves as the mechanosensor in muscle tissue.

Amino acids

Vandenburgh and Kaufman [75] observed that when muscle cells were stretched there was an increase in the uptake of amino acids. This increased amino acid uptake could be completely blocked by the sodium channel inhibitor oubain. Further, ouabain could completely block the stretch-induced activation of protein synthesis [76]. This work has been supported by studies showing that amino acid transport in the soleus and plantaris is increased following synergist tenotomy in normal and hyposectamized rats [77] and 3 hrs following resistance exercise in humans [14]. Together, these data suggest that stretch activation of amino acid influx may function as a mechanosensor in skeletal muscle. If intracellular amino acids are a mechanosensor, then increasing intracellular amino acid content by protein feeding or stimulating protein degradation within the muscle should increase the response to resistance exercise. This has been confirmed in a number of studies showing that protein supplementation in association with resistance exercise has a synergistic effect on protein synthesis rates [17,19,20] and as discussed above a significant correlation exists between the fractional synthetic rate (FSR) and fractional breakdown rate (FBR) within a muscle after a single bout of resistance exercise in humans. Thus, muscle could transduce mechanical stimulation into accelerated protein synthesis by increasing internal amino acid concentration through amino acid transport and activation of proteolysis.

It is unlikely that one single factor is responsible for growth signalling in response to resistance exercise. Under different loading parameters it is possible that a different mechanical sensor is used. Alternatively, one of the mechanosensory pathways may lead to the activation of the others. At the

moment, the costamere appears to be the most likely mechanosensor, however, it will be important to determine whether the costamere phenotypes observed in cardiac muscle are conserved in skeletal muscle.

Like the mechanosensor, the molecular mechanisms governing the growth processes are still controversial. It is clear however, that two key cellular responses are required for hypertrophy: increased protein synthesis [78] and an altered transcriptional profile [77]. Here, we will focus on the kinase mTORC1 and its potential role in regulating protein synthesis and capacity and β-Catenin and micro (mi)RNA for their roles in controlling the levels of families of mRNA within the cell in response to work-induced hypertrophy.

2. Translational control and mTORC1

An increased rate of translation in response to resistance exercise has been observed in humans, rats, chicken, and rabbits. The acceleration of protein synthesis is due to an increase in mRNA activity, the amount of protein produced from a molecule of mRNA. This was first observed by Wong & Booth [79] in rats using electrical stimulation. They demonstrated that up to 48hrs following a single bout of resistance exercise the increase in protein synthesis as measured by incorporation of L-[4,5-3H]leucine was predominantly due to an increased protein translation rate rather than an increase in mRNA. This has since been demonstrated in humans [15] where muscle protein synthesis and mRNA activity are increased 4-24hrs after resistance exercise performed by the biceps brachii. Consistent with these findings, resistance exercise in rats causes a large shift in the polysome profile such that more ribosomes bind to the available mRNA, which indicates enhanced translation initiation [80].

Translational control refers to the control of gene expression through the regulated translation of specific mRNAs to protein. The rate of translation refers to the overall amount of protein produced from the cytoplasmic pool of mRNA. Therefore, when referring to increased translation, this indicates an increase overall protein produced, whereas translational control refers more specifically to increase synthesis from only specific transcripts. Translation is an energy costly process (four high energy phosphates per peptide bond [81]) and as such the rate of protein synthesis must be tightly controlled. The energy status of the cell, endocrine milieu, mRNA content, and the concentration and specific activities of the translational machinery are some factors that control translation either specifically or more generally.

One protein that plays an important role in translation and translational control is mTORC1. mTORC1 coordinates inputs from mechanical stimuli [82], energy status [83] and from endocrine [84] and nutrient [85] signals to regulate

the rate of and capacity for protein translation [86]. mTORC1 regulates translation generally as well as exerting translational control of specific, suppressed mRNA. How mTORC1 is thought to control these processes as well as how each might contribute to accelerated protein synthesis will be discussed below.

Overload hypertrophy is dependent on increased mTORC1 activity [59] and ex vivo models have demonstrated an increase in mTORC1 activity following passive stretch [74,87,88]. Several animal models of resistance exercise have confirmed an increase in mTORC1 activity anywhere from 10mins [89] (rats operantly conditioned to squat) to 6 hrs (stimulation of the sciatic nerve) [80,90,91] into the recovery period. Several different modes of resistance exercise in humans have also been shown to activate mTORC1 with a similar time course [92,93]. Furthermore, in both rats and humans the activation of mTORC1 correlates with muscle hypertrophy and in humans the increase in strength following training (Figure 1; [94]). Due to the central role of mTORC1 in protein translation and regulation by resistance exercise and mechanical stimuli, mTORC1 is thought to be one of the central regulators of hypertrophy.

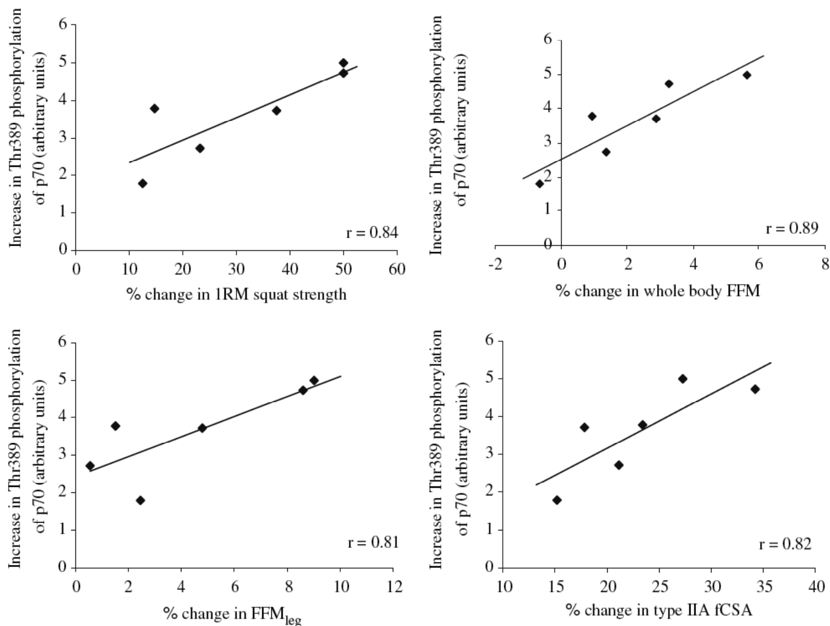

Figure 1. Correlation between S6K1 phosphorylation 30 minutes after resistance exercise and the increase in: (A) strength; (B) whole body and (C) leg fat free mass; and (D) type II fibre hypertrophy (from [94]).

2.1 Overview of translation

Translation of protein results from the integration of three distinct processes: initiation; elongation and termination. Initiation is the assembly of the components of the translational machinery. Elongation is the process by which amino acid residues are added to the C-terminal end of the growing polypeptide chain. Termination is the process by which the peptidyl-tRNA is cleaved releasing the protein. Under normal conditions, ribosomes are stacked 80-100 nucleotides apart along an mRNA. However, they are capable of stacking much closer, up to 27-29 nucleotides apart [95], allowing a 3 fold increase in RNA activity. For this reason it is the initiation phase (in most circumstances) which acts as the rate-limiting step in translation [96]. We will give a brief description of the translation process, for more information on this topic see the following excellent reviews; Kozak, [97], Hershey, [98] and Gingras et al. [96].

The first step of translation initiation is the formation of the 43S preinitiation complex. Central to this process is eIF2. When bound to GTP, eIF2 primes the 43S ribosomal subunit for mRNA binding. eIF2 is therefore critical for the translation of all mRNA. The second step is the association of mRNA with the 43S ribosomal subunit. This step is catalysed by eIF4E binding to the 5' cap of the mRNA [96] and its association with eIF4G within the 43S complex. The association of eIF4E with eIF4G, and thus the mRNA with the 43S ribosomal subunit, is regulated by a competitive inihibitor, the eIF4E binding protein (4EBP). When hypophosphorylated, 4EBP binds to eIF4E and prevents the association with eIF4G and translation of the mRNA. Phosphorylation of 4EBP relieves this inhibition and permits initiation. The third step of initiation is the melting of the secondary structure of the 5' end of the mRNA, scanning for the initiation codon, and formation of the 80S ribosome. Unwinding and scanning of the mRNA is catalysed by eIF4A and eIF4B and is essential for the translation of mRNA with large amounts of secondary structure in their 5' untranslated regions such as growth factors and protooncogenes. Binding of the 60S is promoted by the hydrolysis of eIF2·GTP to GDP and the release of eIF2 from the preinitiation complex. This results in the formation of the 80S ribosome and the onset of protein synthesis.

2.2 mTORC1 and cell size regulation

Many of the processes detailed above are regulated by the activity of mTOR. mTOR is a member of the phosphatidylinositol kinase (PIK) related kinases [99], although it does not possess lipid kinase activity and is instead an efficient serine/threonine protein kinase [100]. The activity of mTOR is dependent upon several adapter proteins GβL [101], raptor [102], rictor [103], Sin1 [104] and Proctor/PRR5 [105,106], which form two separate

mTOR complexes capable of regulating distinctive pathways. The mTOR complex 1 contains GβL, raptor and mTOR and is rapamycin sensitive. GβL functions to stabilise the association between mTORC1 and raptor and enhances the kinase activity of mTORC1 towards its targets [117], however it is not essential for mTORC1 activity [107]. Raptor is an adapter protein that identifies and binds substrates that contain TOS (TOR signalling) motifs [108] such as 4EBP and S6K1 [109]. mTORC2 on the other hand consists of mTOR, GβL, Sin1 and Proctor/PRR5 and is rapamycin insensitive [103]. GβL again is essential for the association between rictor and mTOR, which is essential for the kinase activity of the complex [104,107]. Sin1 is also required for this association and kinase activity [104]. The role of Proctor/PRR5 appears less clear. While its expression is regulated by rictor, it associates with the mTORC2 complex, and it participates in growth factor signalling down stream of mTORC2 [106], it is not essential for mTORC2 complex formation or function [105]. mTORC2 regulates distinct targets from mTORC1, for instance mTORC2 is proposed to act upstream of the Rho GTPases [110], modulates the phosphorylation of PKC [103] and PKB [111], and has a role in regulating the cellular cytoskeleton [103,110]. As the regulation of mTORC1 by resistance exercise and overload is better characterised than mTORC2 we will focus on mTORC1 in the regulation of cell size and protein synthesis.

Much of our knowledge about the role of mTORC1 in the regulation of translation and cell size has come from genetic studies and the use of rapamycin in drosophila, mice, yeast and tissue culture systems. Rapamycin treatment of yeast [112] drosophila [113], and mammalian cells [114] causes cell cycle arrest, reduces cell size in drosophila [113] and mammalian cells [115], and inhibits cap dependant translation [116] and ribosomal biogenesis [117]. Elegant and thorough work by Fingar et al. [115] demonstrated the critical role mTORC1 plays in regulating cell size through S6K1 and 4EBP. These authors overexpressed various mutants of mTORC1, S6K1, eIF4E and 4EBP in a range of mammalian cell types with and without the mTORC1 inhibitor, rapamycin and the PI3K inhibitor, LY294002. Using flow cytometeric analysis to measure cell size, and biochemical analysis of downstream signalling events they showed that in all cell types, and cell cycle phases rapamycin blocked activation of S6K1, phosphorylation of 4EBP and increased association of 4EBP with eIF4E resulting in reduced cell size. The reduction of cell size was modest, approximately 10% and overall cellular protein content was reduced by approximately 30%. Overexpression of rapamycin resistant (RR) mTORC1 in the presence of rapamycin rescued the effect of rapamycin on signalling and cell size, however, neither RR-S6K1 nor eIF4E alone elicited a similar rescue. Co-expression of both RR-S6K1 and eIF4E together elicited a stronger rescue, however, full rescue was not achieved.

Transcriptional profiling of yeast [118] and mammalian cells [119] treated with rapamycin revealed that TOR/mTORC1 also controls the transcription of ribosomal genes. mTORC1 can regulate transcription by the three classes of RNA polymerases [Pol-I (controls rRNA synthesis), Pol-II (controls ribosomal protein genes) and Pol-III (controls 5S rRNA synthesis)] which control the transcription of ribosomal components [120]. In cultured drosophila cells, rapamycin, RNAi and transcriptional profiling identified regulators of ribosomal biogenesis, and ribosomal assembly as genes regulated by dTOR [113]. Interestingly, as knock down of dIF4E and dS6K together did not reduce cell size to the same extent as rapamycin, this suggests that TOR has other outputs that control ribosomal biogenesis and cell size [113]. Together, these data demonstrate that mTORC1, through S6K1, 4EBP, as well as other unknown effectors, plays an important role in regulating cell size and cell cycle progression by exerting effects on translation initiation and the rate of ribosome biogenesis.

2.3 S6K1

As discussed above, mTORC1 controls cell size largely through S6K1 and eIF4E/4EBP. S6K1 is a member of the AGC family of serine/threonine kinases that are dependent on phosphoinositide dependent protein kinase-1 (PDK1) for activation [121]. While S6K1 can be phosphorylated by a number of other kinases, including mitogen activated protein kinase (MAPK) and protein kinase-C (PKC), it is dependent on mTORC1 [122] and PDK1 [123,124] for full activity. mTORC1 associates with S6K1 through raptor [109], which not only binds to the TOS motif in S6K1 but also suppresses an inhibitory function in the C-terminal motif (RSPRR) of S6K1 allowing for phosphorylation at Thr389 (mTORC1 site) and Thr229 (PDK1 site) [125]. Mammalian cells express two splice variants of S6K1 termed p85S6K [126], predominantly localised to the nucleus [127], and p70S6K, which is predominantly cytoplasmic [127]. There also exists a separate homologue of S6K1 referred to as S6K2, which is the product of a separate gene but does not appear to regulate cell size [128].

Knock out of S6K1 in mice [128] and dS6K in drosophila [129] results in a small organism phenotype, the transgenic mice or drosophila being ~15% smaller than controls. Analysis of cells cultivated from these organisms reveals that the reduced body size is a consequence of a reduced cell size, as cell number and proliferation remains the same [128,129]. Skeletal muscle cells cultured from $S6K1^{-/-}$ mice were approximately 20% smaller and were unable to increase myotube diameter normally in response to IGF-1 and constitutively active PKB [130]. Park *et al.* [131] made similar observations in C_2C_{12} cells treated with rapamycin or overexpressing mutants of mTORC1

and S6K1. These experiments demonstrated that S6K1 was required for growth factor-induced skeletal muscle hypertrophy in vitro.

The requirement for S6K1 may result from its role in regulating initiation, elongation, the capacity for translation, and/or nuclear export. S6K1 regulates elongation indirectly by phosphorylating and inhibiting elongation factor 2 kinase (eEF2K) [132]. In basal conditions, eEF2K phosphorylates and inactivates eEF2 slowing elongation [133]. S6K1 removes this inhibition promoting translocation in the step phase of the ribosome [134]. However, since elongation is not the rate limiting step of translation [135], increasing the rate of elongation alone will not significantly increase the rate of protein synthesis without a concomitant increase in initiation [135].

S6K1 can regulate initiation directly through eIF4B [136,137]. eIF4B promotes the unwinding of the 5'-untranslated region (UTR) of mRNA [138-140]. Phosphorylation of eIF4B by S6K1 increases its activity. When non-phosphorylated eIF4B or a non-phosphorylatable S6K1 are overexpressed, eIF4B binding to the initiation complex and translation are inhibited, suggesting that the ratio of non-phosphorylated to phosphorylated eIF4B may provide a mechanism for controlling translation. This is especially true for mRNA containing a high degree of secondary structure in their 5'UTR such as growth factors and protooncogenes [81]. While eEF2 and eIF4B are clearly regulated by S6K1, they are not independent regulators of cell size and are not exclusive targets of S6K1.

The best studied mechanism proposed for how S6K1 may regulate translation and cell size is through the selective upregulation of the translational machinery [141]. All ribosomal proteins characterised contain a region in their 5'UTR that is rich in pyrimidines (5'TOP) [142]. In growth-arrested cells, these transcripts are selectively repressed, but in growing cells or upon mitogen stimulation, these transcripts shift to the polysome fraction [143], thus increasing the expression of the translational machinery and the protein synthetic capacity of the cell. In a series of elegant experiments Jefferies et al. [143] and Terada et al. [117] demonstrated a correlation between mTORC1 activity, S6K1 activation, S6 phosphorylation and the translation of 5'TOP transcripts. Jefferies later showed [144] that a dominant negative S6K1 could repress the translation of 5'TOP transcripts in a similar manner to rapamycin and that a rapamycin resistant S6K1 could rescue cells from the effects of rapamycin. These data strongly suggested a model whereby phosphorylation of S6 by S6K1 improved the translational efficiency of the transcripts containing a 5'TOP.

However, the role of S6 phosphorylation in 5'TOP translation has recently been questioned [145-147]. Work from the Meyuhas lab utilizing the PI3K inhibitor LY294002 and rapamycin suggested that S6K1 and S6

phosphorylation were not required for the improved translation of 5'TOP transcripts [146,147]. However, the most convincing evidence against this model has come from transgenic mice. Disruption of either S6K1 or S6K1 and 2 resulted in no difference in the rapamycin-sensitive effects of mitogens on S6 phosphorylation or the translation of 5'TOP transcripts [128,148], proving that S6K1 is not required for 5'TOP regulation. Furthermore, knock in mice expressing a non-phosphorylatible form of S6 were still able to promote the translation of 5'TOP RNAs [145]. Therefore, although increased S6K1 activity can selectively promote the translation of RNA containing a 5'TOP, the underlying mechanism is currently unknown.

Another potential way S6K1 could regulate ribosomal biogenesis is by regulating the transcriptional activity of upstream binding factor (UBF) [149,150]. UBF binds to human selectivity factor-1 (SL1) and enhances the activity of the ribosomal DNA (rDNA) transcriptional machinery, increasing the ribosomal content of the cell [151]. S6K1 is required for growth factor-induced increases in UBF phosphorylation and rDNA transcription [149]. However, no evidence for a direct interaction between UBF and S6K1 has been found. Zhang et al. [150], have also shown that PKB/mTORC1/S6K1 signalling regulates RNA polymerase-1 dependent transcription [144], suggesting another important role for S6K1 in regulating ribosomal biogenesis.

The last way that S6K1 may control translation is by regulating nuclear export of mRNA. The only factor to date that is a specific target of S6K1 and not S6K2 is the Aly/REF like protein, SKAR (S6K1 Aly/REF like target) [152]. While the molecular function of SKAR has yet to be elucidated, the homology between SKAR and Aly/REF suggests that it might be involved in mRNA splicing or possibly mRNA export [153].

To summarise, through S6K1, mTORC1 regulates protein initiation through eIF4B phosphorylation, elongation through eEF2K phosphorylation, ribosomal biogenesis at the level of rDNA transcription through regulation of UBF and 5'TOP translation, and potentially regulates mRNA processing and export through SKAR.

2.4 eIF4E - 4EBP

Like S6K1, eIF4E can control translation through the regulation of initiation, ribosomal biogenesis, and/or the export of mRNA from the nucleus. Unlike S6K1, overexpression of eIF4E alone can lead to cellular transformation or aberrant growth in a number of cell lines [154-156] and higher levels of eIF4E are found in a range of cancers [157]. Knock down of eIF4E with expression of antisense RNA reduces global translation rates and lengthens cell division [158]. Together, these data suggest that, like S6K1, eIF4E is an important regulator of cell growth.

Regulation of eIF4E occurs at two levels; direct phosphorylation and sequestration by the 4EBPs. Phosphorylation of eIF4E is associated with growth and activation of translation, but the mechanism depends on the site of phosphorylation. Phosphorylation by PKC or protease activated kinase II (a ribosomal S6 kinase) at Ser53 increases the association of mRNA and eIF4E with the 43S preinitiation complex and increases translation initiation. Phosphorylation at Ser209 by MAPK-activated protein kinase (MNK) on the other hand promotes growth by stimulating the export of growth-related mRNA (such as ornathine decarboxylase (ODC) [159], vascular endothelial growth factor (VEGF) [160], and cyclin D1 [161,162] from the nucleus. However, while knockout of MNK1 and MNK2 eliminates eIF4E phosphorylation at Ser209, this does not alter cell growth or development in mice. In drosophila by contrast, phosphorylation of eIF4E on Ser251 (equivalent to mammalian Ser209) is absolutely required for normal growth and development [163]. These data show that eIF4E phosphorylation can lead to growth in an mTORC1-independent manner. However, whether phosphorylation is an essential control mechanism in mammals remains to be demonstrated.

A better characterized mechanism for regulating eIF4E activity is through the 4EBPs. 4EBP1/2 bind to and inhibit the function of eIF4E [164] in an mTORC1-dependant manner [116,165-170]. The interaction of the 4EBPs with eIF4E is dependant on a conserved sequence of 12 amino acids that is found also in eIF4G, suggesting that the non-phosphorylated form of 4EBP competitively inhibits the binding of eIF4E to eIF4G. mTORC1 phosphorylates 4EBP in response to insulin and other growth stimuli and this phosphorylation leads to a reduction in the interaction between eIF4E and 4EBP thus allowing eIF4E to bind eIF4G and promote 43S preinitiation complex formation.

Liberation of eIF4E from the 4EBPs might also promote the translocation of eIF4E into the nucleus. Recently, a nuclear import protein called the eIF4E transporter (4E-T) has been identified and cloned [171], suggesting an active shuttling of free eIF4E into the nucleus. Translocation of eIF4E to the nucleus might be important in growth since mutation of Trp73 on the dorsal surface of eIF4E prevents binding of either eIF4G or the 4EBPs, but retains the capacity to increase cyclin D1 mRNA in the cytoplasm and transform cells [172]. If free eIF4E is required for this growth effect, then mTORC1 would regulate this through phosphorylation of 4EBP. This has been suggested in L6 cells where growth factor-induced hypertrophy results in increased expression of cyclin D1 in a rapamycin sensitive manner [173].

While these data suggest an important role for 4EBP in controlling growth, in contrast to the S6K transgenic mice, knockout of 4EBP1 alone [174] or 4EBP1 and 4EBP2 [175] does not lead to increased animal size or

tumour formation. In fact loss of 4EBP leads to a small animal phenotype in male mice [174], and the double knockout results in accelerated diet-induced insulin resistance [175]. Both of these findings can be explained if the 4EBPs act as competitive inhibitors of S6K1. Raptor binding to 4EBP decreases the basal activity of S6K1. In the absence of the 4EBPs, S6K1 activity increases leading to feedback inhibition of the insulin receptor substrates (IRS; discussed below) and insulin resistance.

2.5 mTORC1 and resistance exercise

Baar and Esser were the first to suggest a role for mTORC1 in muscle hypertrophy [80]. They demonstrated an increase in S6K1 phosphorylation, as measured by gel shift analysis, from 3-36 hrs after a single bout of resistance exercise. More interestingly, the degree of hypertrophy following 6 weeks of training correlated with the degree of S6K1 phosphorylation 6 hours following resistance exercise [80]. These findings have been confirmed by a number of independent groups in a number of different models from rat to man. Not only is S6K1 activity increased following electrical stimulation, but also by ablation overload [59], passive stretch ex vivo [88] operant conditioning in rats [176], and resistance exercise in humans [93,177]. Interestingly, the correlation between S6K1 phosphorylation and hypertrophy in rats has also been confirmed in humans. The increase in strength, fat-free mass, and fibre cross-sectional area after 14 weeks of training all correlated with the phosphorylation of S6K1 30 minutes after a single bout of resistance exercise [94]. This indicates that S6K1 is a great marker for muscle growth and suggests that mTORC1 activation might be important in the hypertrophic response.

Unlike S6K1, changes in eIF4E phosphorylation have never been demonstrated in response to resistance exercise [176]. On the other hand, the mTORC1 dependant phosphorylation [176] and inhibition of 4EBP increases significantly in response to resistance exercise in the rat [89,178]. Synergist ablation overload of the rat plantaris shifts the association of eIF4E from 4EBP to eIF4G in a rapamycin sensitive manner [59]. Furthermore, the mTORC1 dependent increase in cyclin D1 expression during skeletal muscle hypertrophy in vitro suggests that the liberation of eIF4E from 4EBP may also promote growth by increasing eIF4E in the nucleus.

Together, these data suggest that mTORC1 regulates work-induced muscle growth through S6K1 and 4EBP/eIF4E. These proteins selectively increase the translation of important growth response genes such as cyclin D1 [173], eIF2Bε [179], and ribosomal proteins [144] resulting in an overall increase in translation and ribosomal biogenesis (Figure 2). Repeating the exercise stimulus at a sufficient frequency would result in greater protein accretion and muscle hypertrophy.

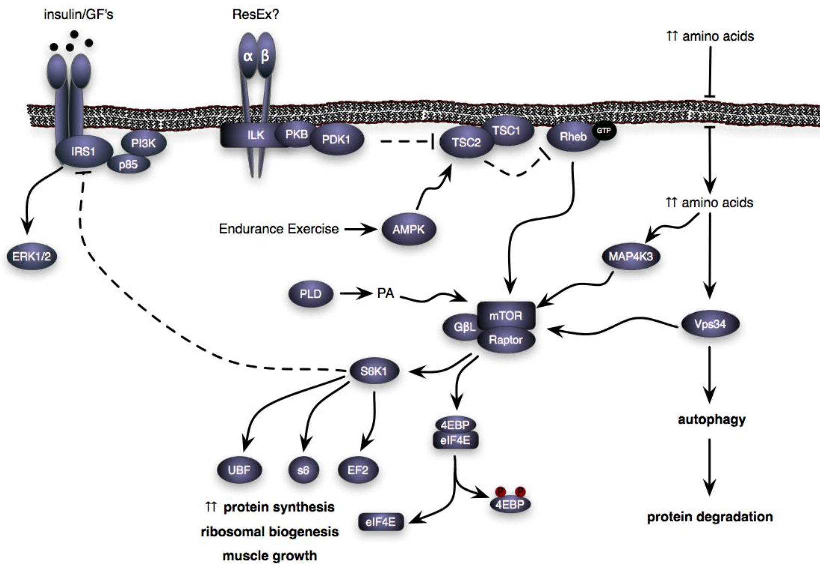

Figure 2. Schematic representation of the activation of mTORC1 by resistance exercise. Note that the activation of mTORC1 by integrin-linked kinase-induced PKB phophosylation and/or MAP4K3 are at this point hypothetical. However, the role of the other factors including phosphatidic acid and Vps34 have been seen experimentally.

3. Regulation of mTORC1

3.1 Endocrine regulation of mTORC1

Skeletal muscle expresses and responds to a range of endocrine signals. As we have previously discussed, skeletal muscle expresses a range of growth factors in response to load. Recently a variety of cytokines, termed myokines, have been found to be expressed by skeletal muscle in response to exercise [180,181]. Several of the myokines have been implicated in mediating muscle growth. For instance IL6 (interleukin 6) [182], IL4 [183], and IL15 [184-187] have all been implicated in muscle growth and IL6 [182], and IL15 [188] are regulated following load or exercise. However, due to space limitations we will focus on the mode of activation of mTORC1. For instance mTORC1 is one of the primary mediators through which insulin [189] and IGFs [130,131,190] exert their influence on anabolism. β2-adrenergic agonists such as clenbuterol [191] are also capable of activating mTORC1 and inducing muscle growth. Evidence also suggests a role for Wnt signals in the regulation of mTORC1 [192] suggesting that mTORC1 serves as a control point for many of the growth factors that affect muscle size.

Growth factor regulation of mTORC1

Canonical insulin and growth factor signalling begins with ligand binding to a membrane receptor [193,194]. The insulin and IGF-1 receptors are hetero-tetrameric proteins consisting of two identical α-subunits that protrude into the extracellular space anchored to the membrane by their association with the two identical β-subunits that project into the intracellular space. The β-subunits possess intrinsic tyrosine kinase activity, which is stimulated upon ligand binding to the α-subunits resulting in auto phosphorylation of the intracellular subunits on tyrosine residues. Tyrosine phosphorylation recruits scaffolding proteins such as members of the insulin receptor substrate (IRS) family and Shc (Src homology collagen) proteins which themselves become tyrosine phosphorylated [195,196]. The IRS proteins recruit class I PI3Ks to the membrane via the SH2 (Src homology 2) domain in the p85 subunit of the PI3K complex leading to the generation of phosphatidylinositol-3,4,5-triphosphate (PIP3) from phosphatidylinositol-4,5-biphosphate (PIP2) in the plasma membrane [197]. The increase in PIP3 leads to the recruitment of phosphoinositide dependant kinase (PDK)1 and PKB/akt to the membrane through their plekstrin homology (PH) domains, which bind PIP3. Binding of PIP3 leads to a conformational change in PKB allowing it to be phosphorylated by PDK1 at Thr308 [121,198] and mTORC2 on Ser473, resulting in a fully active kinase [111]. PKB can then directly activate mTORC1 [199] through phosphorylation at Ser2448 [200], or indirectly activate mTORC1 by destabilising the inhibitory tuberous sclerosis complex (TSC)1/2 through phosphorylation of TSC2 [201]. The TSC1/2 complex acts as a GTPase activating protein (GAP) towards the small GTPase Rheb (Ras homologue enriched in brain) [202]. When Rheb is in the GTP bound state it acts through an unknown mechanism to activate mTOR [202-204]. Receptor tyrosine kinase phosphorylation also leads to the activation of the Ras/Raf/MAPK pathway [195], which can also inhibit TSC1/2 and activate mTORC1 through RSK [205]. To summarise, growth factors interact with membrane bound receptors and initiate a series of signalling cascades leading to either the direct phosphorylation and activation of mTORC1 or the inhibition of TSC1/2 GAP activity resulting in a greater Rheb-GTP and subsequent mTORC1 activation.

3.2 Energy status

Seminal work by Hickson, [206] demonstrated that concurrent training to improve strength and endurance inhibited strength gains, and actually led to a decline in strength after ten weeks. The possible causes for this are well reviewed by Nader, [207], but here we will discuss how energy status can signal to regulate mTORC1 activity. A key enzyme involved in energy sensing

is the adenosine monophosphate dependent protein kinase (AMPK). AMPK senses cellular energy status by responding to the ratio of AMP/ATP [208]. When cellular AMP concentration increases, AMP binds to AMPK, allosterically activates the enzyme, and makes it a better substrate for its upstream kinases LKB1 or the calcium calmodulin-activated protein kinase kinases (CaMKK) α and β [209-211]. The upstream kinases phosphorylate AMPK at Thr172 leading to full activation [212]. Active AMPK inhibits anabolic processes and activates catabolic processes that enhance ATP generation [213]. One of the key anabolic processes that is inhibited by AMPK is protein synthesis. Activating AMPK using the AMP mimetic 5-aminoimidazole-4-carboxamide 1-beta-d-ribonucleoside (AICAR) in rats decreases protein synthesis by reducing mTORC1 signalling [214]. The molecular mechanism for this was elucidated by Inoki et al. [215] who showed that under conditions of energy stress, TSC2 was phosphorylated and activated by AMPK thus inhibiting mTORC1 activity and decreasing protein synthesis. Therefore, any process that leads to energy stress can lead to the activation of AMPK and subsequent inhibition of mTORC1 and protein synthesis. One physiological stimulus that leads to the activation of AMPK is endurance exercise [216] and the activation of AMPK is thought to be one of the key molecular mechanisms underlying the adaptation to endurance exercise [217]. As a result, endurance exercise likely decreases the hypertrophic response by activation of AMPK and a subsequent reduction in the activation of mTORC1.

Energy stress in the form of hypoxia is also capable of modulating mTORC1 activity. Hypoxia inhibits overall translation rates and increases the association of eIF4E with 4EBP1 [218] by modulating mTORC1 activity. The decrease in mTORC1 activity is the result of an increase in expression of the hypoxia inducible gene REDD1 [219]. REDD1 inhibits mTORC1 by activating TSC1/2 [220] in an AMPK-independent manner [218]. Interestingly, following treatment with protein synthesis inhibitors, mTORC1 activity is rapidly increased. This increase in mTORC1 appears to be due to the rapid degradation of REDD1 following the treatment with inhibitors of protein synthesis [221]. The degradation of REDD1 results in TSC1/2 inhibition and mTORC1 activation. A similar model is possible for hypoxia. At the cessation of hypoxia, REDD1 would be rapidly degraded potentially leading to mTORC1 activation. This might explain recent work showing a positive effect of resistance training with venous blood flow restriction [222]. Although hypoxia is not normally a consequence of resistance training, very light resistance exercise during hypoxia results in skeletal muscle hypertrophy in both rats and humans [223]. Interestingly, following training in the hypoxic state there is a disproportionately large increase in mTORC1 activity [224]. One possible explanation for the ability of light exercise and hypoxia to

activate mTORC1 is that exercise in the hypoxic state results in a large increase in REDD1 during the exercise bout resulting in inhibition of protein synthesis. Immediately following the hypoxic exercise, there is a rapid decrease in REDD1 leading to a compensatory increase in mTORC1 activity after the exercise bout driving an increase in protein synthesis and muscle hypertrophy.

3.3 Nutrient regulation

Increased muscle size and strength are optimised by the combination of resistance exercise and appropriate nutrition. Integration of the two stimuli likely occurs at the level of mTORC1. However, how amino acids work upstream of mTORC1 remains to be fully elucidated. Following a single bout of resistance exercise, both protein synthesis and protein degradation are increased [225]. In the fasted state, the protein balance is negative with protein breakdown exceeding protein synthesis. This balance is partially recovered following resistance exercise due to an increase in protein synthesis [13,14,226]. However, in the fasted state there is a concomitant increase in protein degradation so the net balance remains negative. Net balance becomes positive only when nutrients are added. Here, it is the addition of protein, specifically essential amino acids that has the strongest effect. It also appears that the timing of the supplement also plays a role in the determination of net protein balance. Ingestion of an amino acid-carbohydrate mix immediately before or within one hour of an exercise bout greatly enhances the degree of protein synthesis and reduces breakdown thereby maximising muscle growth [20,227,228].

The mechanisms by which amino acid signalling induces cell growth differ depending on the amino acid studied [229]. Some amino acids induce cell swelling indirectly through an increase in cell osmolarity following sodium ion dependent amino acid transport. Another group, including amino acids like leucine promote cell growth by activating protein synthesis [230-232]. Leucine is a branched chain amino acid (BCAA) along with valine and isoleucine. BCAAs, particularly leucine, are very potent anabolic agents and effectively increase the activity of mTORC1 [233]. Rapamycin partially inhibits the ability of leucine to increase protein synthesis. The partial inhibition suggests an as yet unidentified mTORC1 independent pathway [230,234]. Recently, two amino acid sensitive pathways have been identified upstream of mTORC1. The first is the class 3 phosphoinositol kinase Vps34 identified by Byfield and colleagues [235]. Vps34 can be inhibited in the absence of amino acids and may be permissive in the activation of mTORC1. Vps34 also plays a role in protein degradation in the form of autophagy with its binding partners Vps15 and Beclin1 [236,237]. This suggests that Vps34 might underlie the correlation between protein degradation and protein

synthesis observed by Phillips and his colleagues [225]. The second potential amino acid sensor is mitogen-activated protein kinase kinase kinase kinase (MAP4K)-3. Like Vps34, MAP4K3 is activated by the administration of amino acids [238]. MAP4K3 is also required for the activation of mTORC1 and when MAP4K3 is overexpressed in HEK-293 cells there is an increase in mTORC1 activity. Interestingly, MAP4K3 knockdown results in a decrease in cell size suggesting that it can directly affect the anabolic state within cells.

Amino acid consumption in parallel with resistance exercise can maximise muscle growth [20,228]. As reviewed by Tipton and Witard [239] both the timing and the type of amino acids also affect post exercise protein synthesis. Most of the data suggests that amino acid uptake into muscle and the subsequent increase in protein synthesis is highest when the supplement is consumed immediately before or within an hour of completing exercise. The effect of the timing might be the result of a window of increased amino acid uptake associated with resistance exercise. Indeed, amino acid uptake is increased to a greater degree when the amino acids are taken before the exercise bout, likely due to the shunting of blood to the active muscle. Furthermore, muscle stretch results in the activation of amino acid uptake [76] and this increase in amino acid uptake is required for the activation of protein synthesis [75]. This suggests that resistance exercise promotes amino acid uptake and this increase in amino acid uptake is important for the activation of mTORC1, either though Vps34 or MAP4K3, and skeletal muscle hypertrophy.

3.4 Mechanical stimulation

As discussed above, amino acids enhance protein synthesis in response to stretch but do not appear to initiate mTORC1 activation. Little is known about how mTORC1 is initially activated by mechanical stimuli. Wortmannin, a potent inhibitor of PI3K, has no effect on stretch activated p70S6K(T389) phosphorylation suggesting that PI3K is not required [87,88]. Even though PI3K is not activated by stretch, PKB/akt, a downstream target of PI3K, can be activated immediately after resistance exercise/high frequency stimulation of muscle [59,240,241]. Therefore, there is either a PI3K-independent mechanism for activating PKB/akt or the activation of PKB/akt is not required for mTORC1 activation. One PI3K-independent mechanism for activating PKB/akt, through the integrin-linked kinase, has been described above. Hornberger and his colleagues [88] have suggested an alternative mechanism for mechanically-induced signalling through mTORC1. First they showed that mTORC1 is still activated in $PKB^{-/-}$ mice. In addition, using whole muscle in-vitro stretch they demonstrated that autocrine/paracrine factors did not activate mTORC1, suggesting that PKB/akt was not required for mTORC1 activation. In a follow up study, they described a sustained increase (15-90

minutes post) in the lipid second messenger phosphatidic acid (PA) in response to mechanical stimulation [242], confirming work performed some 17 years previous [243]. Two chemically distinct inhibitors of phospholipase D (PLD), the enzyme that catalyses the production of PA and choline from the phosphotydlcholine, blocked the mechanical activation of mTORC1 and the production of PA in response to stretch. These data suggest PLD forms part of the pathway from mechanical stimulation to mTORC1. Indeed, PA has already been proposed as a critical signalling molecule in cardiac hypertrophy [244] and stretch can lead to rapamycin-independent activation of mTORC1 [242]. This occurs since PA competes with rapamycin for binding to the FRB domain of mTOR [245]. There are, however, several other enzymatic pathways that regulate PA both positively and negatively that may play a role in the activation of mTORC1. One is diacylglycerol kinase (DGK), the enzyme that converts diacylglycerol (DAG) into PA [246,247]. Another, is phospholipase A (PLA) the enzyme that reduces PA to lysophosphatidic acid and free fatty acids through deacetylation [248]. The last is the PA phosphatase (PAP) that converts PA into DAG [249]. However, the involvement of these enzymes has yet to be investigated in the mechanical regulation of mTORC1. It is clear that PA can activate mTORC1 following mechanical stretch, but it remains to be definitively determined how this occurs.

3.5 Negative feedback

Tremblay & Marette [250] were the first to demonstrate a negative feedback loop in the insulin signalling pathway. They found that incubating L6 cells in high concentrations of amino acids for 1 hour could reduce insulin-induced 2-deoxyglucose (2-DG) uptake. This effect was caused by increased IRS-1 phosphorylation and subsequent degradation, leading to decreased PKB activity. This response was rapamycin sensitive suggesting that mTORC1, or a mTORC1 target caused it. Because the response was correlated to S6K1 phosphorylation they hypothesised that it was mediated by S6K1. Consistent with this, loss of dTSC1/2 in drosophila leads to a disruption in insulin signalling which is rescued by RNAi against dS6K [251]. Later work by Harrington et al. [252] in $TSC2^{-/-}$ (tuberous sclerosis complex-2) mouse embryonic fibroblasts (MEFs), which have constitutive mTORC1 signalling, confirmed these earlier findings. This study utilized mutants of S6K1 and IRS-1 to demonstrate a direct interaction between S6K1 and IRS-1 both at the protein and mRNA level. Microarray analysis of $TSC2^{-/-}$ and wild type MEFs showed that IRS-1 mRNA was downregulated in the absence of TSC2. This was confirmed with qPCR, and IRS-1 mRNA was partially rescued with the use of rapamycin or RNAi against S6K1. IRS-1 protein was also suppressed, and this was rescued by re-expression of TSC2.

Next they screened fragments of IRS-1 using in vitro phosphorylation assays with S6K1 and identified Ser302 as an in vitro phosphorylation site for S6K1. TSC2$^{-/-}$ MEFs had higher IRS-1 Ser302 phosphorylation, and S6K1 RNAi reduced this. Finally they showed that the S6K1 mediated phosphorylation of IRS-1inhibited IRS-1 function and prevented growth factor mediated signalling, thus further confirming the function of S6K1 as a negative regulator of insulin signalling. More recently IRS-1 has been shown to be a direct target for mTORC1 through the association of IRS-1 with raptor again resulting in an inhibition of IRS-1 function [253]. This series of experiments helps to explain the phenotype of 4EBP/2$^{-/-}$ mice, discussed above, and also that of mice overexpressing IGF-1. Mice overexpressing IGF-1 under the control of a myosin light chain promoter display hypertrophied muscle with increased mTORC1/S6K1 phosphorylation and reduced PKB phosphorylation [72]. Few studies have examined the effect of resistance training on this negative feedback loop. However some have analysed the effect of resistance training on insulin sensitivity, which is a good read out for the activity of this loop. 6 weeks of overload-induced hypertrophy of the Extensor Digitorim Longus has no effect on insulin mediated glucose disposal in rats [254] and long term resistance training in young [255], and diabetic [256] subjects improves insulin responsiveness. However there are confounding results regarding the acute response. Howlett et al. [257] found a reduction in insulin signalling immediately after a single bout of resistance exercise, this was associated with a reduction in insulin stimulated IRS-1 tyrosine phosphorylation which could suggest an increased serine phosphorylation [252]. Koopman et al. [258] found that a single session of resistance exercise increased insulin sensitivity measured 24 hrs post stimulation, however the mechanism is unclear. Although this feedback loop is clearly important in nutrient regulation it is still unclear whether it is physiologically relevant to resistance training or hypertrophy.

4. Transcriptional control

Prior to the current focus on protein synthesis and mTORC1 activity, a great deal of early research focused on the importance of transcription in the development of skeletal muscle hypertrophy. The absolute requirement for de novo transcription in the development of skeletal muscle hypertrophy was demonstrated using the transcriptional inhibitor actinomycin D to prevent overload-induced hypertrophy of the plantaris in rats [77]. However, the specific transcriptional events that are required for muscle growth remain unknown. Part of the challenge in determining the transcriptional regulation of muscle hypertrophy is the greater complexity of this aspect of growth in comparison to translational regulation. This complexity is the result of the fact that the acute changes in RNA synthesis can be very different than the

long-term changes in the expression profile within muscle [259]. Since the acute changes associated with resistance exercise are thought to cause muscle hypertrophy, this area has been more extensively studied. Most of these studies including: Carson et al. [260] studying the effects of 3 days of overload on the transcriptional profile in the soleus of rats; Chen et al [261] studying the transcriptional changes 1-6hrs following a single bout of lengthening contractions in rats; and Kostek et al [262] studying the transcription profile in humans 3-24 hours after resistance exercise have shown that genes from functional classifications such as; proliferation, autocrine/paracrine signalling, extracellular matrix, immune response, metabolism, and protein synthesis/degradation are effected by acute resistance exercise. The most striking finding from these studies is that resistance exercise alters the expression of not just individual genes but large clusters with functions as diverse as inflammation [263], growth and differentiation [261,264], and protein degradation [265]. This suggests either that there are specific transcription factors that can target large gene clusters, or that the expression of these genes is coordinately controlled by global transcriptional regulators like microRNAs.

Some transcription factors that are required for the activity of muscle-specific promoters have been identified [266]. These include the myogenic regulatory factors: Myf5, MyoD, myogenin, and MRF4, the myocyte enhancing factor (MEF)2, serum response factor (SRF), and transcription enhancer factor (TEF)1. The myogenic transcription factors myogenin [267-269], MyoD [268,269] and MRF4 [269] are increased during load-induced hypertrophy. In response to overload in the chicken, both SRF and TEF1 increase and this leads to an increase in skeletal α-actin expression [270,271]. The increased expression of β-myosin heavy chain (MHC) [272] during overload hypertrophy in the plantaris muscle of rats is dependent on an A/T rich element [273] and TEF1 binds to this element and can upregulate the expression of the β-MHC gene [274] suggesting that SRF and TEF1 might be involved in the hypertrophic response. However, none of these genes have been shown to be necessary for skeletal muscle hypertrophy.

A few transcriptional regulators are either sufficient or necessary for skeletal muscle hypertrophy. Furthermore, these factors control the expression of gene clusters making them likely candidates for mediating the transcriptional response to load-induced skeletal muscle hypertrophy. As a result, we have chosen to focus our attention on these factors, specifically myostatin/β-catenin and microRNAs.

4.1 Myostatin / β-catenin

Myostatin is a member of the transforming growth factor β (TGF) family that acts through the regulation of cell cycle genes [275], the myogenic

regulatory factors MyoD [276,277], and myogenin [277] to promote myoblast proliferation and suppress differentiation [275,278]. Myostatin initially gained interest when it was discovered to be the cause of the double muscle phenotype seen in Belgian Blue cattle [279]. Without the influence of myostatin during development, muscle growth is almost unrestrained and the result is massive muscle hypertrophy in dogs, sheep, cattle [279,280] and humans [281]. For more in depth reviews on myostatin and TGFβ signalling see excellent reviews by Lee [282], Massague [283] and Tsuchida et al. [284]. Briefly Myostatin exerts a transcriptional influence through an interaction with various membrane receptors such as the ActRIIB (activin receptor type IIB) that ultimately leads to the phosphorylation of receptor regulated Smads (R-Smads). When phopsphorylated, the R-Smads, in association with the common mediator Smad4, translocate to the nucleus where they bind to the Smad binding element and together with a variety of coactivators control the transcription of various genes. The importance of this signalling pathway in the determination of muscle size is evidenced by the fact that genetic manipulation at various points in the pathway results in a hypertrophic phenotype. For instance overexpression of a dominant negative ActRIIB receptor, Follistatin (which inhibits myostatin function) [285] and Ski (which inhibits Smad function) [286] all induce skeletal muscle hypertrophy.

Whether myostatin plays a key role in load-induced hypertrophy in humans remains controversial. One study demonstrated that myostatin transcription is down regulated 24hr after a single bout of resistance exercise in young men and women, but the response is blunted in the aged [287]. Another study demonstrated a reduction in the myostatin receptor (ActRIIB) with no change in myostatin 1hr post resistance exercise [288]. Interestingly, basal myostatin expression was increased by 21 weeks of resistance training, but after training myostatin was acutely depressed 48hr after a single bout of resistance exercise [288]. In rats, overload suppresses myostatin expression at 3 days (rapid growth phase) but returns to normal levels by 21 days of overload (slower growth phase). The response was retained in hyposectamized animals suggesting it is independent of pituitary hormones [289]. One long-term hypertrophy study in humans tried to correlate myostatin expression to the degree of hypertrophy achieved after 16 weeks of resistance training [290]. They assessed the degree of hypertrophy achieved by 66 subjects and assessed myostatin expression after the first and last training bouts according to whether the subjects were extreme, modest or non-responders. They showed that the change in myostatin expression did not correlate with the development of hypertrophy.

One problem with interpreting the myostatin studies is that few of these studies assess myostatin activity. Most of this work measures myostatin mRNA changes with resistance exercise, which may not accurately depict

myostatin signalling. As described above, this pathway is regulated at a number of different levels including myostatin level, receptor binding, receptor level, and Smad signalling. Therefore, measuring the amount of myostatin mRNA within a muscle is not sufficient to determine the activity of this pathway. For instance, genomic analysis after resistance exercise has identified extracellular matrix proteins as being increased. Since myostatin plays an important role in the expression of ECM in muscle, this suggests that myostatin signalling may be enhanced following exercise. Hopefully the recent identification of Mighty as a direct target of myostatin activity [322] will give us an accurate marker of the activity of the myostatin pathway following resistance exercise and will definitively answer whether the myostatin pathway is decreased by resistance exercise. There is no question that myostatin regulation is essential for muscle development in both the pre- and post-natal period, but the contribution of myostatin to skeletal muscle hypertrophy has yet to be determined.

Genome-wide analysis of the myostatin knockout mouse identified the Wnt/β-catenin signalling pathway as being disproportionately upregulated in the absence of myostatin. β-catenin is a multifunctional protein that participates in cell adhesion and transcriptional regulation [291] and is an important component of several signalling pathways. β-catenin is at the centre of two separate pathways with important roles in myogenesis: Wnt, which regulates transcription during development [292]; and m-cadherin, a membrane associated protein that regulates cell adhesion [293,294]. When Wnt signalling is activated, as occurs developmentally and in cancer [295], β-catenin is dephosphorylated as a consequence of the inhibition of its upstream kinase glycogen synthase kinase (GSK)3 and is able to translocate to the nucleus and regulate transcription by association with a range of adapter proteins [296]. Cadherin-catenin interactions are believed to link the cadherins to the actin cytoskeleton [297] and deregulation of β-catenin at the membrane results in defects in myofirbrillogenesis [298]. When β-catenin becomes transcriptionally active, it upregulates the cell cycle regulators cyclin D1 [299,300] and c-myc [299,301]. As discussed earlier cyclin D1 may have an important role in skeletal muscle hypertrophy [173] and inducible overexpression of c-myc in heart tissue in vivo induces cardiac hypertrophy [302].

The Wnt mediated function of β-catenin is positively regulated by intense endurance exercise in rats [303] and humans [304], and both the Wnt and cadherin functions are regulated during overload-induced hypertrophy in rats and mice [305,306]. The co-localization of β-catenin with M-cadherin during overload hypertrophy in rats is increased [305]. Overload of the mouse plantaris also increases the expression of several proteins in the β-catenin pathway, including frizzled, dishevelled-1 and lymphocyte

enhancement factor (LEF-1) [306]. There was also an increase in the association of GSK3 with its inhibitor FRAT resulting in stabilisation of β-catenin, nuclear translocation, and association with LEF-1 in the nucleus. The increased β-catenin-LEF-1 association resulted in a corresponding increase in the expression of the transcriptional targets c-myc and CyclinD1 [306]. An independent microarray study also identified the upregulation of the Wnt receptor Frizzled after 3 days of overload in rat soleus muscles suggesting that the activation of Wnt/β-catenin is a universal response to load-induced hypertrophy [260]. In support of this hypothesis, utilizing an adenovirus to knockdown β-catenin in mice, Armstrong et al. demonstrated that β-catenin is essential for overload-induced hypertrophy (Figure 3; [307]). These data strongly support a role for β-catenin in hypertrophy and raise the possibility that myostatin and β-catenin may act antagonistically on muscle mass by regulating a subset of genes that are critical for muscle growth. However, the identity of these genes remains to be determined.

Recently, the interaction between the Wnt/β-catenin pathway and mTORC1 has been demonstrated both in cells and in human tumours. Inoki et al. [192] were the first to show that Wnt signalling could activate mTORC1 by regulating the phosphorylation and activity of TSC2. In the absence of

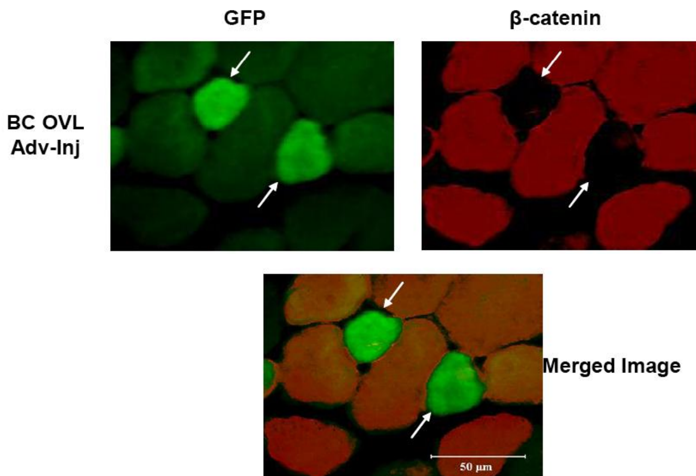

Figure 3. The requirement for β-catenin in skeletal muscle hypertrophy is seen in the lack of growth in fibres where viral knockdown of this protein has occurred. Viral infection is seen as green cells, while β-catenin is stained red. Note the smaller size of the β-catenin null cells after overload (from [307]).

Wnt, GSK3 phosphorylated and activated TSC2. The addition of Wnt to Rat1 fibroblasts resulted in sequestration of GSK3, decreased TSC2 phosphorylation, and increased mTORC1 activation. Interestingly, a follow up study in tumours from tuberous sclerosis patients, where mTORC1 activity is elevated, showed that activation of mTORC1 led to increased β-catenin levels and elevated c-myc and cyclin D1 expression [308]. This suggests that a positive feedback mechanism might exist between mTORC1 and Wnt/β-catenin signalling and this may play a role in the development of skeletal muscle hypertrophy.

4.2 microRNA

While the transcription factors discussed so far bind to the promoter region of their target genes and together with coactivators regulate transcription, micro (mi)RNAs use a much different molecular mechanism to control the expression of large families of mRNA. miRNA are non-coding short chains of mRNA approximately 22nt long. They bind to complementary sequences in the 3'UTR (untranslated region) of target transcripts repressing the translation and promoting the degradation of these mRNAs [309]. To date, the mir-Base catalog of miRNAs contains 5395 entries across 36 different species [310], with approximately 800 predicted in the human genome [311]. Remarkably these miRNAs are estimated to regulate up to 30% of all human genes [312]. miRNA processing is required for normal development [313] and, when overexpressed, miRNAs have the potential to vastly alter the transcription profile of a cell. For instance when the muscle specific mir-1 is overexpressed in HeLa cells the expression profile, as measured by microarray, is shifted towards that of skeletal muscle [314]. Several studies have shown muscle specific miRNAs to be regulated during, and important for, muscle development. For instance, the muscle specific microRNAs mir-1 [315], mir-206 [316] and mir-181 [317] are induced during in vitro myogenesis in C2C12 myoblasts and promote muscle differentiation by downregulating the expression of HDAC4 (histone deacetylase-4), connexin43 [318], and Hox-A11 respectively. mir-133 is on the same cistron as mir-1 and is also strongly induced during myogenesis, but strangely is antagonistic, in that it promotes myoblast proliferation by suppressing SRF (serum response factor) [315]. The expression of both mir-1 and mir-133 is controlled by MEF2 and the myogenic regulatory factors through regulatory elements upstream of the biscistronic miR-1-1/133a-2 message [319]. Interestingly, in response to overload hypertrophy in mice the expression of the unprocessed pri-mir-1, pri-mir-133a and pri-mir-206 are increased. However, the mature mir-1 and mir-133a are suppressed while mir-206 remains unchanged (Figure 4; [320]). The regulation of mir-133a in response to overload is interesting as SRF signalling is required for the regulation of

skeletal α-actin gene during stretch-induced hypertrophy of the chicken anterior latisumus dorsi [270,321]. This data suggests that miRNAs could be involved in altering the transcription profile required for hypertrophy. This hypothesis is extremely interesting as the modulation of a single miRNA can vastly alter the transcription profile of the cell and it is possible that alterations in a few miRNAs could be responsible for the large shift in transcription profiles seen after resistance exercise.

While the possibility exists that miRNAs are involved in the regulation of muscle size following resistance exercise, how resistance exercise regulates the expression of specific miRNAs is still completely unknown. However, the increased interest in understanding the mechanisms underlying miRNA regulation should mean that a number of tools will quickly come online to directly study this question in vivo.

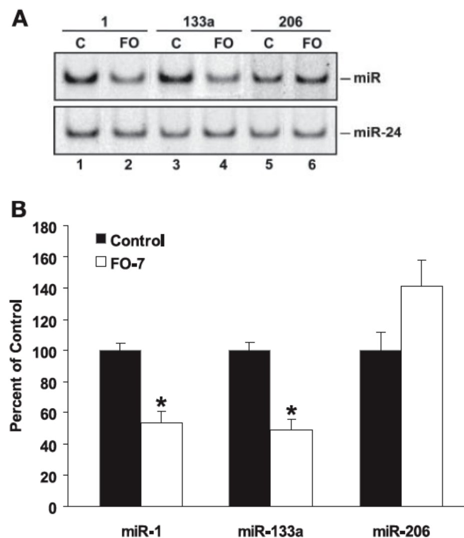

Figure 4. A decrease in the muscle-specific miRNAs mir-1 and 133a following 7 days of functional overload (from [320]). Note also the trend towards increased mir-206 that might be important in the shift from fast to slow muscle fibres.

5. Future perspectives

The last 40 years of research have given us an understanding of some of the key players in resistance exercise-induced skeletal muscle hypertrophy but, we still do not know the extent that each contributes to

in vivo muscle growth. However, the field of skeletal muscle hypertrophy is expanding rapidly, with new techniques and technologies available old problems are now accessible. In the final section we will present some of the most pressing questions that remain and set out a methodological paradigm for investigating the role of a particular factor in the development of skeletal muscle hypertrophy.

1. How is mTORC1 activated following resistance exercise?

As discussed above, how mTORC1 is activated in response to resistance exercise is still controversial. In the heart it seems clear that costameres play a role in the mechanical link between stretch and mTORC1 since ILK [55], melusin [56,57], and the muscle LIM domain protein [58] all co-locate with protein kinase B/akt in costameres and are required for mechanosensing. Whether a similar system is used in skeletal muscle has yet to be determined. Interestingly, in all of the microarray studies performed following resistance exercise the muscle LIM domain protein is upregulated. This suggests that the costameric structure is affected by resistance exercise and may play an important role in mechanotransduction.

2. Does the activation of mTORC1 lead to skeletal muscle hypertrophy?

Clearly, the activation of mTORC1 correlates with muscle hypertrophy. However, experiments designed to demonstrate that mTORC1 can drive skeletal muscle hypertrophy have been unsuccessful [59]. This may indicate that mTORC1 is not sufficient for muscle hypertrophy or it could indicate that the feedback inhibition of IRS-1 and PKB prevents muscle cell growth. In order to determine the role of mTORC1, a transient expression system is necessary. In this way, the effect of repeated transient activation of mTORC1 on muscle cell size can be determined.

3. Does mTORC1 activation lead to β-catenin activation?

As described above, tumours from tuberous sclerosis patients have increased β-catenin levels suggesting that the activation of mTORC1 can lead to the accumulation of β-catenin. Since mTORC1 activation and β-catenin signalling are the two pathways that appear to be necessary for skeletal muscle hypertrophy, mTORC1-dependant activation of β-catenin would provide a unifying molecular mechanism for skeletal muscle hypertrophy. Therefore, the affects of acute activation of mTORC1 on β-catenin levels in muscle in the presence and absence of rapamycin need to be determined.

4. Is myostatin signalling regulated following resistance exercise?

As described above, the role of myostatin in load-induced skeletal muscle hypertrophy remains uncertain. This is predominantly due to the fact the myostatin signalling is controlled at multiple levels and it is difficult to know what to measure. With the identification of Mighty as a specific myostatin pathway target gene [322], this gene can be used as a marker for the activity of the whole pathway. As a result, the expression of Mighty following resistance exercise will be a definitive measure of the activity of mysostatin and will finally answer this basic question of muscle adaptation.

5. Does suppression of mir-1 and/or mir-133a lead to skeletal muscle hypertrophy?

The observation that mir-1 and 133a levels go down following resistance exercise presents the hypothesis that these miRNAs drive the transcriptional changes leading to muscle hypertrophy. This can be directly tested in vitro by knocking down the miR-1-1/133a-2 bicistronic transcript in muscle cells in vitro and determining whether this results in an increase in myotube size.

6. How does resistance exercise alter miRNA levels?

The expression of miRNAs is controlled by specific transcription factors binding to the flanking DNA and increasing the transcription of the miRNA. MEF2 and MyoD sites have already been identified within the upstream region of mir-1 and mir-133a. Of these, MyoD is known to be upregulated following resistance exercise [323-325]. This correlates with the increase in primir-1 and 133a following overload. However, levels of the mature mir-1 and 133a decrease suggesting that the processing of these miRNAs is regulated following resistance exercise. The identity of the processing factor and its control following resistance exercise is of extreme interest if mir-1 and/or mir-133a drive skeletal muscle hypertrophy.

7. How do we identify novel factors that regulate skeletal muscle hypertrophy?

Human and animal models of resistance exercise can be used to screen for factors by looking for changes in signalling cascades that are exercise-specific. However, this will require large-scale investigation of differential phosphorylation following resistance or endurance exercise using proteome-scale screening techniques. Without such techniques, the discovery of factors that are activated by resistance but not endurance exercise will rely on luck.

For instance, the discovery that S6K1 undergoes a prolonged phosphorylation in response to resistance exercise but not endurance exercise was made while investigating the role of 4EBP in exercise [80]. In the future, quantitative mass spectroscopy of proteins from control, resistance exercised, and endurance exercised muscle may well identify novel proteins that play an important role in the adaptation to resistance training. However, these experiments will be quite costly and will require diligent post hoc confirmation to show that the signalling dynamics of the identified proteins are consistent with one or more of the processes involved in hypertrophy and that when the activity of the protein is altered this has the appropriate effect on hypertrophy.

8. Can tissue culture support the identification and characterisation of hypertrophic factors?

Tissue culture models have been extensively used to analyse specific signalling molecules in skeletal muscle. These models traditionally used transient transfection to overexpress the protein of interest and determine its effect on muscle size. One of the problems with these studies is that they use supraphysiological levels of the protein of interest and never do the important confirming experiments such as: (1) expressing of an inhibitor resistant protein that rescues the effect of the inhibitor; (2) expressing a dominant negative form of the protein that can inhibit growth; and (3) expressing downstream targets to recapitulate the phenotype and circumvent inhibitors. Further, care must be taken when modelling hypertrophy in tissue culture. For example, PKB has been clearly demonstrated to play a major role in growth factor-induced hypertrophy in culture [326], can induce muscle growth in vivo [59], and can prevent unloading-induced atrophy [327]. However the signalling dynamics of PKB in response to resistance exercise suggest that long-term activation of PKB is not required for muscle hypertrophy in vivo [90,91]. This is an important point to consider when studying transgenic animals: continuous activation of a signalling molecule may induce growth, but this does not definitively mean that it is important in work-induced muscle hypertrophy.

9. What constitutes in vivo verification of hypertrophic proteins?

The wide use of knock in and knock out mice models has identified proteins that can modulate skeletal muscle size. Indeed, if a gene is absolutely required for muscle hypertrophy, then knocking it out or knocking in a dominant negative form should completely block load-induced skeletal muscle hypertrophy. One example of this was the knock in of a dominant

negative IGF receptor [71]. It was long thought that growth factors produced locally by muscle in response to mechanical stimulation were partly or wholly responsible for hypertrophy, however even though these animals were growth factor resistant they still underwent normal overload-induced hypertrophy. If a gene is proposed to prevent skeletal muscle hypertrophy then knocking it out should relieve an inhibition. An example of this is that of the tumour suppressor LKB1. It was hypothesised that this gene exerted a negative effect on muscle growth by activating AMPK [210], which inhibits mTOR [215]. However, loss of this gene had no effect on overload-induced muscle growth in mice as a consequence of compensation from another AMPK kinase [328]. This illustrates the importance and the drawbacks of relying on genetically modified mice. In this example, it allowed us to identify crosstalk between pathways. In other cases, muscle-specific knockout of a gene from the onset of muscle creatine kinase or α-skeletal actin expression, results in large-scale cellular adaptations that can compensate for the loss of a specific gene. These adaptations are largely non-physiological and introduce confounding variables that prevent proper analysis of the gene of interest. Some of this compensation can be overcome by knocking in genes with mutations in sites of posttranslational modification such as phosphorylation, neddylation or sumoylation rather than knocking them out and this may reveal the physiological relevance of these sites in response to resistance exercise.

10. How can we screen for clinical potential?

When assessing the clinical potential of a pharmacological target it is essential that the outcome measure is force production. Muscle hypertrophy is the consequence of increased protein turnover in favour of protein accretion. Degradation is still a component of the response so that architectural remodelling can occur. Simply inducing growth may not be enough to improve force output. Increasing muscle force production is the result of adaptations within the muscle and the muscle ECM that allows force transduction. The only way to assess if a treatment is effective is to measure the force output of the muscle either in vivo, or in tissue culture with 3D muscle constructs. It is important to remember that having a bigger muscle mass without improved force generating capacity would result in greater disability due to the greater mass that would need to be supported.

Summary

The molecular mechanism of skeletal muscle hypertrophy is a complex interaction between translation and transcriptional events leading to accelerated accretion of protein. Over the past 10 years, key signaling factors that control these processes have been identified. Further, there appears to be

considerable crosstalk between these molecular pathways, suggesting that a common unifying process may underlie the phenotypic changes following resistance exercise. However, identification of this factor is beyond the current work in this area. The search for the unifying mechanism of muscle hypertrophy has been hampered by the fact that there are many roads that lead to increased muscle size and the current literature does not discern between resistance exercise-induced hypertrophy and developmental or growth factor-induced muscle growth. While some commonality likely exists between the pathways it is the differences that will be the key to understanding how to pharmacologically increase muscle mass without causing growth related diseases such as cancer. There is still a lot to learn in this field and a great deal of therapeutic potential to be exploited. Due to the complexity of the signaling it is likely to be some time before effective pharmacological therapies to increase muscle mass are developed, for now the best approach is a good regime of resistance exercise in combination with well timed nutrition.

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