University of Jyväskylä
Department of Biology of Physical Activity

MYOSTATIN AND RELATED PROTEINS IN THE CONTROL OF SKELETAL MUSCLE MASS AND CAPILLARY DENSITY.

Bernardo Moreira Soares Oliveira

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Department of Biology of Physical Activity
University of Jyväskylä
Supervisors: Juha Hulmi and Heikki Kainulainen

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ABSTRACT

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Skeletal muscle wasting is a feature of many pathological conditions such as muscular dystrophies, cancer and diabetes. Human ageing also results in the progressive loss of muscle mass and strength, a condition known as sarcopenia (or myopenia). Therefore, interventions that can reverse or slow down muscle loss are highly desirable. The TGF-β member myostatin is a well-known inhibitor of skeletal muscle growth, but it may also, if deleted, decrease muscle oxidative capacity. We have used the activin receptor 2B (ActR2B) fused to the Fc region of immunoglobulin G (ActR2B-Fc) as a trap to sequester myostatin and inhibit its activity. We sought to evaluate possible differences between doses and frequency of injection, as well as the signalling pathways involved in the response.

For this purpose, healthy C57-BJ mice (n = 5-6 per group) were injected with either 5 or 10 mg/kg ActR2B-Fc once or twice a week for 2 weeks. PBS-injected mice served as controls. Gastrocnemius cryosections were immunostained for sarcolemma (caveolin 3) and capillaries (isolectin). Image analysis (fibre cross-sectional area (CSA) and capillary density) was done using ImageJ. Protein synthesis was measured by puromycin incorporation, and protein expression and phosphorylation were measured by western blotting.

After 2 weeks of treatment we found increased muscle masses and fibre CSA irrespective of dose and frequency of injection. Muscle capillary density (per area) decreased dose-dependently. To study the mechanisms involved mice were injected with a single (10 mg/kg) dose of ActR2B-Fc one or two days before sacrifice. Muscle rpS6 phosphorylation, a marker of mechanistic target of rapamycin (mTORC1) activity, increased 2 days after injection. Accordingly, muscle protein synthesis increased ~30% 2 days after injection. The phosphorylation status of ERK, a MAPK member involved in angiogenesis, decreased in muscle 2 days after injection. These signalling responses were no longer observed after 2 weeks. In conclusion, the present study showed that 2 weeks of ActR2B-Fc administration increased muscle mass and fibre CSA, while reducing capillary density and oxidative metabolism. Combining ActR2B antagonism with other interventions that may attenuate the reduction in capillary density and oxidative metabolism (such as exercise) may yield more desirable outcomes.

Keywords: myostatin, TGF-β, activin receptor 2B, skeletal muscle, hypertrophy.

1. INTRODUCTION

Skeletal muscle is the most abundant tissue in the human body, comprising around 40% of adult body weight. Skeletal muscle itself is approximately 70 % water, 28 % proteins and 2 % minerals and other elements. Skeletal muscle mass is determined by the balance between protein degradation and synthesis. Each of these conditions can be induced by many internal and external stimuli such as diet, exercise, hormones and disease, for example. (Goodman et al. 2011b)

Maintenance of skeletal muscle mass is paramount to our health. Skeletal muscle contraction generates force that allows the performance of daily activities; skeletal muscle is the tissue with the highest metabolic demand and also the largest reservoir of glucose (as glycogen) and amino acids, which is relevant in conditions such as diabetes and obesity; and muscle contraction acts as an anabolic stimulus to bone. (Harridge 2007; Goodman et al. 2011b) Hence, a dramatic decrease in muscle mass affects the whole organism. Many diseases induce muscle atrophy and the degree of muscle loss is related to morbidity and mortality (Jespersen et al. 2006; Zhou et al. 2010). Therefore, the development of new strategies to maintain or increase muscle mass is of clinical interest. However, the ultimate goal is not only to increase muscle mass, but rather to have improved muscle function. Therefore, it is of interest that the whole muscle structure is improved and preserved, including capillaries, extracellular matrix, tendon and its attachment to bone.

Increased muscle mass can be achieved by increasing protein synthesis or inhibiting protein degradation. Myostatin is a well-known and powerful inhibitor of skeletal muscle protein synthesis. Since its discovery myostatin has been studied as a potential therapeutic target against myopathies . One approach to the problem has been blocking its action by soluble binding proteins, one of which is activin receptor 2B (ActR2B).

Therefore, the purpose of this work was to evaluate the effects of an exogenously administered soluble ActR2B (ActR2B-Fc) on skeletal muscle and the possible mechanisms involved in the response. We sought to evaluate possible differences between doses (5 or 10 mg/kg) and frequency of injection (once or twice a week) during a 2-week period. Furthermore, signalling responses and protein synthesis were analysed 1 or 2 days after a single 10 mg/kg injection of ActR2B-Fc.

2. SKELETAL MUSCLE GROWTH AND ATROPHY

Skeletal muscle is a highly plastic tissue that can adapt in different ways. The type and degree of adaptation are specific to and determined by the stimuli received by skeletal muscle. Endurance exercise, for example, is known to induce angiogenesis, increased expression and activity of oxidative enzymes and mitochondrial biogenesis. On the other hand, resistance exercise induces muscle hypertrophy and increased expression and activity of glycolytic enzymes. To achieve these different responses the stimuli must affect different signalling pathways which will ultimately lead to the expression or repression of specific genes, thus altering the cellular architecture and phenotype. (Harridge 2007; Alberts et al. 2008)

In order to increase skeletal muscle mass the rate of protein synthesis must be higher than the rate of protein breakdown. Protein synthesis encompasses basically three steps: (1) DNA transcription into a messenger RNA (mRNA), (2) mRNA translation into a peptide chain, and (3) post-translational modifications that result in a functional protein. Protein synthesis, therefore, can be regulated at any of these steps. (Alberts et al. 2008)

The main pathway thought to be involved in skeletal muscle atrophy is the ubiquitin-proteasome system (UPS), the other one being the autophagy/lysosome pathway (Zhao et al. 2008; Glass 2010; Goodman et al. 2011). The UPS involves the tagging of proteins for their subsequent degradation by the proteasome system (Sandri 2010). A polyubiquitin chain is covalently attached to the proteins, a process that is regulated by an enzyme cascade consisting of ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3) (McFarlane et al. 2006). The polyubiquitin chain is recognised by the 26S proteasome complex and the proteins are then degraded. Subsequently, the amino acids from the degraded peptide chain are released to be used by the cell. It is noteworthy that the degradation apparatus may target not only proteins, but also mRNAs and the translation machinery, therefore interfering with protein synthesis. (Szewczyk & Jacobson 2005)

The skeletal muscle tissue has a pool of stem-like cells called satellite cells (SC). These cells are located between the basal lamina and the sarcolema and are involved in the regeneration and hypertrophy processes. Upon muscle injury or trauma they are activated and undergo proliferation, and later fuse with adjacent fibres to restore muscle

function. (Harridge 2007; Tiidus 2008) The fusion of SC with muscle fibres provides additional myonuclei and therefore increases the cellular transcriptional capacity. Ageing reduces the ability to activate SC and this contributes to decreased muscle mass and strength (Snijders et al. 2009; Thornell 2011). Muscle dystrophies are known to prematurely exhaust the SC pool due to repeated degeneration/regeneration cycles, which can contribute to progressive muscle loss (Kottlors & Kirschner 2010).

2.1 Insulin-like growth factor 1, Akt and mechanistic target of rapamycin (mTOR)

The main signalling pathway regulating muscle protein synthesis is the insulin-like growth factor 1 (IGF-1)/Akt/mTOR pathway. IGF-1 is a strong hypertrophic agent and its over-expression leads to muscle hypertrophy, whereas its knock-out or depletion leads to severe atrophy. IGF has different splicing variants (isoforms), namely IGF-1A, IGF-1C and IGF-1E, the latter also known as mechano growth-factor (MGF). Insulin can also bind the IGF-1 receptor and therefore activate this pathway. Binding of IGF-1 to its receptor leads to phosphorylation of the insulin receptor substrate (IRS), which in turn induces the formation of phosphatidylinositol triphosphate (PIP3), a membrane phospholipid. PIP3 acts as a docking site for Akt (also known as PKB) to be phosphorylated. Phosphorylated Akt then acts as a kinase to phosphorylate or dephosphorylate many substrate targets, one of which is the mammalian target of rapamycin (mTOR). Akt activates mTOR by phosphorylating and thus inhibiting tuberous sclerosis complex (TSC), an inhibitor of mTORC1. Parallel to mTORC1, activated Akt inhibits glycogen synthase kinase 3β (GSK-3β), thus releasing eukaryotic initiation factor 2B (eIF2B) to participate in the translation initiation process. (Harridge 2007; Goodman et al. 2011a) Akt may also have a role in determining the number and size of type 2 fibres. Akt1 deficiency reduced EDL cross-sectional area (CSA) but not soleus'. However, the number of type 2 fibres in soleus was reduced. (Goncalves et al. 2010) An overview of the IGF-1/Akt/mTOR pathway is shown in figure 1.

mTOR is a key factor in the regulation of protein synthesis and cell homeostasis. It is in the intersection of key pathways that respond to nutrients, energy availability, growth factors and stress. The function of mTOR is determined by its association with two

complexes termed mTORC1 and mTORC2. mTORC1, which is rapamycin-sensitive, is composed of five proteins: mTOR, regulatory-associated protein of mTOR (Raptor), G protein β-subunit-like (Gβ1), proline-rich Akt substrate 40 kDa (PRAS40) and DEP-domain-containing mTOR-interacting protein (Deptor). mTORC2, which is rapamycin-insensitive, is composed of at least six different proteins: mTOR, rapamycin-insensitive companion of mTOR (Rictor), stress-activated-protein-kinase-interacting-protein 1, protein observed with Rictor-1, Gβl and Deptor. (Zoncu et al. 2011)

The nutrient and energy status of the cell is an important factor modulating mTOR activity. For example, the ratio of AMP to ATP is an important index of energy availability. This ratio is sensed by the AMP-activated protein kinase (AMPK). AMPK can phosphorylate TSC2 thus allowing the TSC1-TSC2 complex to induce the catabolism of Rheb-bound GTP. GDP-bound Rheb is unable to activate mTORC1. Moreover, AMPK phosphorylates RAPTOR thus inhibiting its binding to mTORC1. (Zoncu etl al. 2011) Another factor that can modulate mTORC1 activity is amino acid availability. Amino acids from protein degradation are capable of recruiting mTORC1 to the membrane of lysosomes, and this process is dependent on vacuolar H^+-ATPase (Zoncu et al. 2011b). The ability of branched-chain amino acids (BCAA) to increase protein synthesis seems to rely on mTORC1 activation. Leucine, the BCAA with the most prominent capacity of increasing protein synthesis, activates mTORC1 signalling to induce protein synthesis. (Suryawan et al. 2008) Furthermore, leucine (and other amino acids) act through a distinct pathway than Akt (TSC-independent) to activate mTORC1 (Winter et al. 2011). Other environmental factors such as oxygen availability also influence mTOR activity. Hypoxia impairs mitochondrial ATP generation, thus increasing the AMP to ATP ratio and activating AMPK. Hypoxia also induces the expression of regulated in development and DNA damage response 1 (REDD1). REDD1 also inhibits mTORC1 activity by inducing the formation of TSC1-TSC2 complex. (Zoncu et al. 2011a) Exercise is capable of influencing these factors. During exercise the AMP/ATP ratio increases, which can lead to AMPK activation (Hardie 2008). Also, exercise induces transient hypoxia (at the cellular level), thus activating REDD1 (Murakami et al. 2011) .

mTOR's downstream targets include eukaryotic initiation factor 4E (eIF4E)-binding

protein 1 (4E-BP1) and S6 kinase 1 (S6K1), which control translation initiation and progression. Phosphorylated 4EBP-1 dissociates from eIF4E, thus allowing the latter to take part in the pre-initiation complex composed of eIF3, eIF4F, 40S ribosomal subunit and the ternary complex eIF2/GTP/Met-RNA. Phosphorylated S6K1 activates e.g. ribossomal protein S6 (rpS6), a component of the 40S ribossomal subunit. (Goodman et al. 2011a; Zoncu et al. 2011a) Also, eIF3f has been shown to be an important factor in the control of muscle mass. It is a target of atrogin-1 for polyubiquitination and subsequent degradation. Expression of a mutant eIF3f insensitive to atrogin-1 enhances its hypertrophic effects, and its over-expression induces muscle hypertrophy associated with increased sarcomeric protein content. The mutant eIF3f protected muscle cells against starvation-induced atrophy by maintaining higher levels of phospho-S6K1 and eIF3f. (Lagirand-Cantaloube et al. 2008; Csibi et al. 2010) Csibi et al. (2010) have shown that eIF3f amino acid residues 91-221 (corresponding to the Mov34 domain) are the main ones responsible for its binding to S6K1, whilst the C-terminal domain mediates its binding to mTOR. Interestingly, deletion of the C-terminal domain disrupted the activation of S6K1 by mTOR and resulted in decreased levels of phosphorylated S6K1 and rpS6. Furthermore, they showed that the C-terminal domain contains a TOR signalling (TOS) motif that is important for mTOR function, the FETML motif (amino acid residues 323-327). Mutation of the first residue of this motif led to pronounced inhibition of mTORC1 activity and muscle differentiation. In addition, it also disrupted the interaction between eIF3f and mTOR/raptor. Moreover, over-expression or inhibition of eIF3f altered the rate of cap-dependent translation, which is thought to be a rate-limiting step in protein synthesis.

Given the importance of mTOR to energy homeostasis it's likely that it influences mitochondrial function. Treatment of C2C12 cells with rapamycin (an inhibitor of mTOR) decreased the mRNA content of PGC-1α, a paramount regulator of oxidative metabolism. Rapamycin treatment decreased mitochondrial DNA content by 32% and oxygen consumption by 12%. On the other hand, constitutively active mTOR increased the expression of genes down-regulated by rapamycin. (Cunningham et al. 2007)

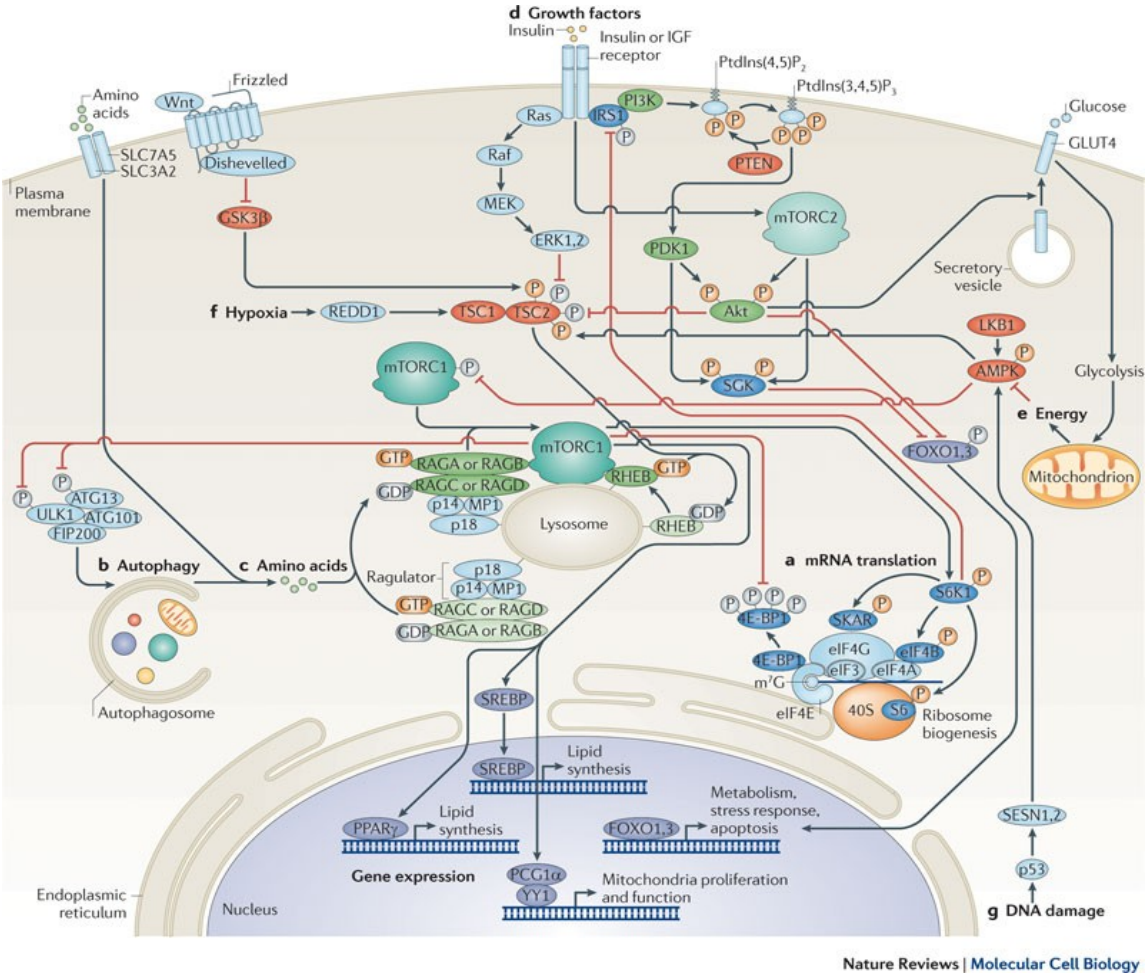


FIGURE 1. Overview of the IGF-1/Akt/mTOR pathway. From Zoncu et al. (2011).

2.2 Mitogen-activated protein kinase

Members of the mitogen activated protein kinase (MAPK) family have been implicated in muscle adaptation to various stimuli. MAPK members are serine/threonine kinases which are involved in the regulation of various and distinct cellular processes such as proliferation, differentiation, mitosis, cell survival and apoptosis. The main members of the MAPK family are extracellular signal-regulated kinase 1/2 (ERK-1/2), c-Jun N-terminal kinase (JNK) and p38, but the presence of at least six MAP kinases in yeast suggests that there are more in mammals. MAPK themselves are activated by phosphorylation of serine and threonine residues by MAPK kinases (MPAKK or MAP2K) and are inactivated by phosphatases. (Kyriakis & Avruch 2012) MAPK are involved in skeletal muscle hypertrophy and their inhibition can blunt IGF-1-induced

hypertrophy (Haddad & Adams 2004). ERK 1/2 inhibition also blunted testosterone-induced hypertrophy in L6 myoblasts (Wu et al. 2010). These researchers found that ERK 1/2 and mTOR were mediators of the cellular response to testosterone, while Akt and IGF-1 were not. ERK 1/2 signalling is also required for Myf5 activation (Perez-Ruiz et al. 2007), suggesting that ERK is involved in skeletal muscle cell differentiation. On the other hand, MAPK also mediates myostatin signalling through JNK (Huang et al. 2007), and in cardiomyocytes myostatin was up-regulated after stress in an ERK-dependent manner (Bish et al. 2010). Bish et al. (2010) argued that myostatin may be part of a negative feedback loop to control muscle mass increase. MAPK members also play a role in angiogenesis (Giuliani et al. 2004; Mavria et al. 2006; Milkiewicz et al. 2007; Pagès 2007). Milkiewicz et al. (2007) showed that ERK 1/2 and JNK were necessary for the expression of VEGF and MMP, respectively; and in myeloma cells ERK inhibition inhibits the secretion of VEGF and thus angiogenesis (Giuliani et al. 2004).

Skeletal muscle hypertrophy has traditionally been looked upon from the point of view of increased muscle mass and myofibrillar protein synthesis. However, it must be ensured that the whole muscle structure is functional and thus the hypertrophic process has to be coordinated to allow the formation of new blood vessels and adaptation of adjacent tissues such as bone and tendon. The larger muscle must be supplied with nutrients and oxygen, as well as removal of waste products and undesired metabolites. Also, the series elastic components must adapt in accordance so that proper force transduction can occur. MAPKs have a role in exercise adaptation, such as mitochondrial biogenesis and angiogenesis (see below).

2. 3 Angiogenesis

Angiogenesis, i.e. the formation of new blood vessels, is a complex process that, like muscle hypertrophy, must be tightly coordinated. The interaction between these two processes must be understood for the proper development of interventions. The effect of physical exercise on both processes is also of interest since exercise is known to induce angiogenesis (Matsakas et al. 2012) and muscle hypertrophy (Hulmi et al. 2007), depending on the exercise modality.

The main factors controlling the formation and adaptation of blood vessels are the vascular endothelial growth factors (VEGF). Members of the VEGF family include e.g. VEGF-A, the main molecule controlling blood vessel morphogenesis, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF). VEGFs regulate chemotaxis, differentiation, proliferation, cellular fusion and remodelling of endothelial cells. VEGFs act mainly through VEGF receptor 2 (VEGFR2), a tyrosine kinase receptor. It is noteworthy in the context of myostatin inhibition that TGF-β seems to influence angiogenesis. Human hereditary haemorrhagic teleangiectasia is a disease characterised by vascular malformations. The TGF-β receptors endoglin and ALK-1 were implicated in the pathogenesis of this condition. (Adams & Alitalo 2007; Carmeliet & Jain 2011)

Angiogenesis may have a role in muscle regeneration. Borselli et al. (2010) examined the effects of combined delivery of IGF-1 and VEGF on ischaemia-induced muscle regeneration. They found that combined delivery of both growth factors led to earlier and more pronounced muscle regeneration. It also prevented the formation of fibrotic tissue and the loss of innervation whilst increasing fibre diameter and muscle force. The delivery of both growth factors or VEGF alone increased blood vessel density compared to control or IGF-1 only. However, it is possible that these effects are specific to the injury model used. (Borselli et al. 2010)

Baligand et al. (2010) did an extensive *in vivo* functional assessment of myostatin-null mice. They found different electrically-stimulated exercise-induced perfusion kinetics between wild-type and myostatin-null animals. The increase in perfusion at the beginning of the exercise protocol was delayed in myostatin-null mice, whereas after exercise they presented prolonged hyper-perfusion. Exercise-induced oxygen extraction was also delayed in myostatin-null animals. Baligand et al. (2010)

Myostatin KO has a negative effect on capillary density, which can be counteracted by physical exercise (Matsakas et al. 2012). However, it is not known whether the rapid muscle growth induced by myostatin blocking alters capillary density in adults.

3. MYOSTATIN

Myostatin, also known as growth and differentiation factor 8 (GDF-8), is a member of the transforming growth factor β (TGF-β) superfamily of growth factors and acts to negatively regulate muscle mass. The myostatin protein was discovered in 1997 by McPherron and Lee during a screening for new TGF-β members (McPherron et al. 1997). Later it was found to be mutated in the cattle breeds Belgian Blue and Piedmontese, which present the double muscling phenotype, indicative of its role in controlling muscle size (Kambadur et al. 1997).

Myostatin possesses all the characteristics of a TGF-β member: a signal sequence for secretion, a proteolytic processing site (RSRR) and a carboxyl-terminal region containing a conserved pattern of cysteine residues (cysteine knot). As its name suggests it is expressed mainly in skeletal muscle with lower levels also present in liver, adipose tissue and heart. Myostatin is synthesised as a 375-amino acid precursor protein which later undergoes two proteolytic cleavages to become biologically active. After the first cleavage myostatin is found in a latent form attached non-covalently to its pro-peptide and with a molecular weight of 40 kDa. The second cleavage yields the mature C-terminal homo-dimer with biological function and a molecular weight of 25 kDa. (Lee 2004) Proteases such as matrix metalloproteases (MMP) and ADAM have been shown to be involved in the cleavage of myostatin (Huet et al. 2001). In skeletal muscle, myostatin seems to be present in an extracellular pool of uncleaved pro-peptide (Anderson et al. 2008). In serum, myostatin circulates bound to related proteins such as the pro-peptide, follistatin-related gene (FLRG, also known as FSTL-3) and growth and differentiation factor-associated serum protein-1 (GASP-1) (Hill et al. 2002; Lee 2010).

Myostatin mRNA is first expressed during embryogenesis by cells of the myotome of the developing somites, and continues to be expressed by skeletal muscle cells during adulthood (Lee 2004). In zebra-fish embryos it was shown to control muscle progenitor cell proliferation (Tee et al. 2009). Accordingly, myostatin knock-out (KO) muscle possesses more fibres than wild-type muscle, consistent with its role in controlling muscle cell proliferation during development (Matsakas et al. 2010). Myostatin was shown to be produced and proteolytically processed in myoblasts (in vitro) (Yamanouchi et al. 2000), which may be an indication of a feedback loop to control

skeletal muscle cell proliferation.

3.1 Myostatin Signalling

As a TGF-β member myostatin activates the canonical TGF-β pathway which signals through Smad proteins to control gene transcription. There are two types of TGF-β receptors which are present as homo-dimers on the cell membrane, type 1 and type 2 receptors. Type 2 receptors include e.g. TGF-β receptor 2, activin receptor 2A, activin receptor 2B, whilst type 1 receptors are the activin-like kinase (ALK) receptors 1-7. TGF-β members bind to a type 2 receptor on the cell surface. Upon binding the ligand-type 2 receptor complex recruits a type 1 receptor, which is then phosphorylated at various Ser/Thr residues by the type 2 receptor. The phosphorylated type 1 receptor enables the recruitment and phosphorylation of the receptor-regulated Smads (R-Smad). The activated R-Smad forms a complex with Smad4, a common mediator of the pathway. This complex then translocates to the nucleus where it is involved in transcription regulation. Myostatin binds to activin receptor 2B (ActR2B) as a type 2 receptor, which then recruits either ALK4 or ALK5 as a type 1 receptor. (Schmierer & Hill 2007) It was recently shown that myostatin recruits preferentially ALK5 in muscle tissue, whilst ALK4 is activated in non-muscle tissues (Kemaladewi et al. 2011). Myostatin seems to act on skeletal muscle mainly in autocrine way. A differential response to either endogenous or exogenous myostatin was shown in muscle cells. The different response was attributed, at least partially, to exogenous myostatin binding follistatin on the cell surface. (Ríos et al. 2004)

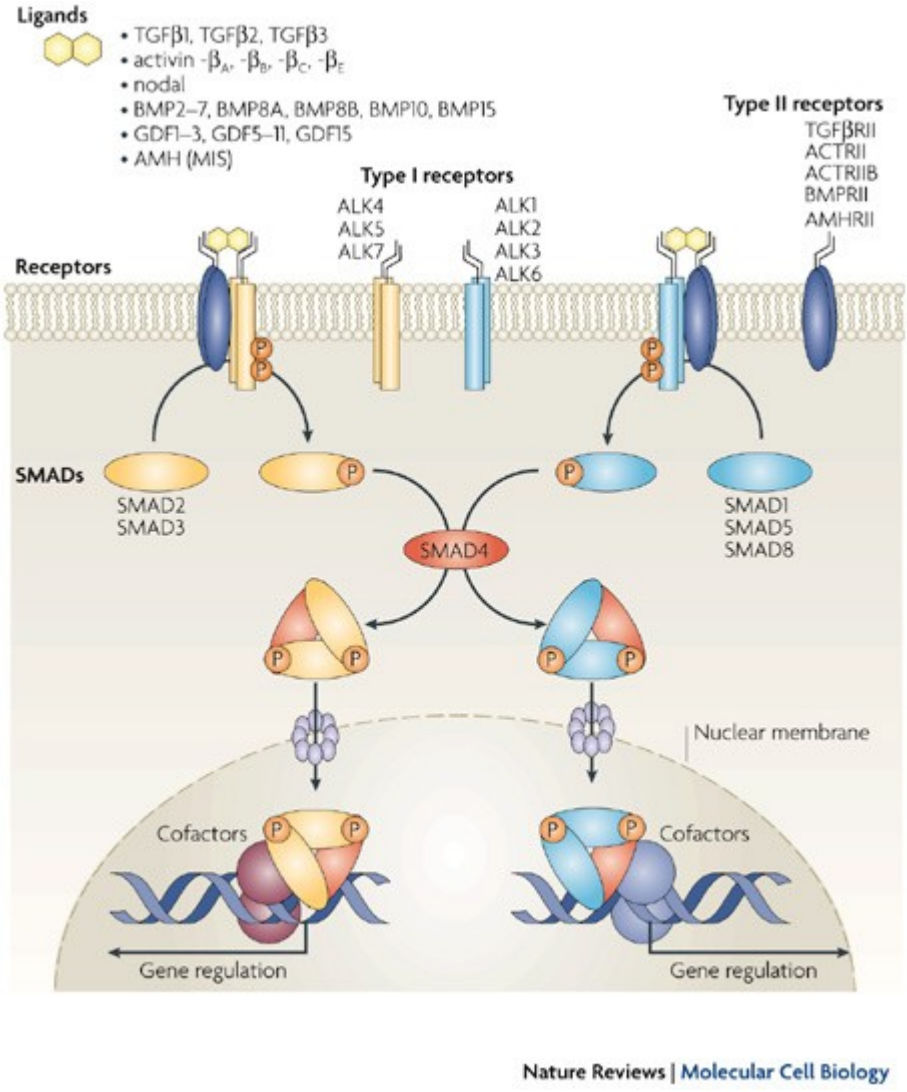


FIGURE 2. Canonical TGF-β pathway. Myostatin (GDF-8) binds to ActR2B and ALK4/5, thus activating Smad2/3. From Schmierer and Hill (2007).

During development myostatin seems to exert its effect on muscle progenitor cells by interfering with cell cycle regulators. The cell cycle is regulated by cyclin dependant kinases (CDKs) and their interaction with associated cyclins. This interaction is affected by CDK inhibitors (CKI). Myostatin was shown to both up-regulate the CKI p21 and down-regulate CDK2. CDK2 and p21 control the G1 to S transition during the cell cycle. Thus myostatin acts to induce cell-cycle arrest and thus inhibit cell proliferation. (Thomas et al. 2000; McCroskery et al. 2003) Myostatin-induced p21 expression was not detectable before 2h of stimulation, with a peak at 4h post-stimulation, indicative of a transcriptional response (Philip et al. 2005). Myostatin-null cells also seem to undergo

an altered differentiation process. McCroskery et al. (2003) reported altered kinetics of differentiation markers (i.e. myogenin and MyoD) between wild-type and myostatin-null myoblasts *in vitro*. After induction of differentiation (by switching medium) MyoD levels rapidly peaked in both cell lines. However, myostatin-null cells presented a delayed decline in MyoD levels. Myogenin levels peaked 6h after induction of differentiation in wild-type cells, whereas in myostatin-null cells the peak was delayed and observed at 24h. Myostatin treatment was also shown to inhibit the expression of MyoD and myogenin (Huang et al. 2007; McFarlane et al. 2011). Trendelenburg et al. (2009) showed that myostatin, along with other TGF-β members (e.g. activin A/AB/B, GDF-11, TGF-β1) inhibit human skeletal muscle cell differentiation, as evidenced by lower fusion index, myotube diameter and creatine kinase activity. They've also presented evidence that both Smad2 and Smad3 participate in inhibition of differentiation. Furthermore, inhibition of both Smads had an additive effect when compared with inhibition of either Smad alone. (Trendelenburg et al. 2009)
MAPK is involved in Smad-independent signalling of myostatin and TGF-β (Philip et al. 2005; Huang et al. 2007; Kyriakis & Avruch 2012). Bish et al. (2010) hypothesised that ERK regulates myostatin expression through MEF-2, since the myostatin promoter contains a MEF-2 responsive site. They found that cardiomyocyte stress (induced by phenylephrine) up-regulated myostatin and induced nuclear translocation of MEF-2, and these responses were blocked by ERK inhibition. The TGF-β activated kinase 1 (TAK1) is an important mediator of TGF-β Smad-independent signalling. It was shown to link myostatin signalling to JNK (Huang et al. 2007) and p38 through (Philip et al. 2005) MKK4 and MKK6, respectively. Furthermore, blocking of both MAPK members blunted myostatin-induced proliferation repression. The effects and especially the time-course of myostatin blocking on MAPK signalling remains to be determined.

3.2 Myostatin and protein synthesis/degradation

The IGF-1/Akt/mTOR pathway is the main regulator of skeletal muscle protein synthesis. Myostatin being such a powerful regulator of skeletal muscle mass is likely to influence this pathway. Myostatin has been shown to down-regulate the Akt/mTOR pathway (Morissette et al. 2009; Trendelenburg et al. 2009; Rodriguez et al. 2011),

therefore reducing muscle protein synthesis. Moreover, myostatin KO muscle presents elevated levels of phosphorylated Akt, mTOR and rpS6 (Morissette et al. 2009; Rodriguez et al. 2011). Despite the importance of Akt signalling for muscle hypertrophy, Akt-KO muscle can still undergo hypertrophy in response to ActR2B inhibition (Goncalves et al. 2010). This response may be mediated by Akt-independent activation of mTOR.

The precise influence of myostatin on protein synthesis and, particularly degradation, is still controversial. Previous works have shown that inhibiting myostatin increases the absolute rate of protein synthesis, but not the fractional rate (Welle et al. 2009; Welle et al. 2011). Myostatin seems to particularly interfere with the translation initiation apparatus to inhibit protein synthesis; cap-dependent translation is especially altered by myostatin (Rodriguez et al. 2011). Rodriguez et al. (20011) reported increased abundance of Akt, mTOR and rpS6 protein in myostatin KO myotubes compared to wild-type. They found no difference in mRNA levels of these proteins, which suggests that the higher protein abundance was due to increased translation. Accordingly, protein synthesis was 21% higher in myostatin KO myotubes with no difference in DNA content. They further reported that myostatin KO myotubes presented higher polysomal to subpolysomal ratio, suggestive of improved translation initiation. The improved translation initiation was accompanied by higher interaction of endogenous eIF4G, raptor and phospho-rpS6 with cap structures, while the interaction of 4E-BP1 to these same structures was less sustained. Likewise, Welle et al. (2009) found an 18.7% higher mean myofibrillar protein synthesis rate in response to JA16 (myostatin neutralising antibody). Interestingly, rapamycin did not alter the response to JA16, suggesting that mTOR may not be the main mechanism through which myostatin affects muscle protein synthesis. Concentrations of S6K, rpS6, 4E-BP1 or Akt were not altered, whereas the phosphorylation state of S6K and rpS6 were increased. Rapamycin treatment abolished the phosphorylation of S6K and rpS6. More studies are needed to elucidate the effects of myostatin blocking on muscle protein synthesis.

The level of protein degradation can be indirectly estimated by the activity of the ubiquitin-proteasome pathway and the expression of muscle specific atrogenes, i.e. atrogin-1 and MuRF1. Once again previous works have shown conflicting results regarding the expression of atrogenes or protein ubiquitination in response to myostatin

(McFarlane et al. 2006; Lokireddy et al. 2011; Welle et al. 2011). McFarlane et al. (2006) found increased $E2_{14k}$ mRNA levels with myostatin treatment *in vitro*, but no difference in protein levels *in vivo*. The expression of MuRF-1 increased *in vivo* but not *in vitro*. They also found increased atrogin-1 expression both *in vitro* and *in vivo*, together with a 60% increase in the amount of ubiquitinated proteins. Inhibition of FoxO1 (with siRNA) blunted the atrogin-1 increase induced by myostatin. On the other hand, Welle et al. (2011) found increased MuRF-1 mRNA at 5 weeks of myostatin inhibition, whilst atrogin-1 mRNA levels did not change. Lokireddy et al. (2011) have shown that myostatin treatment of C2C12 cells leads to a reduction in the level of sarcomeric proteins. Since there was no difference in the mRNA levels of the proteins analysed the reduction was attributed to post-translational modifications such as protein degradation or decreased translational capacity. They found increased mRNA and protein levels of atrogin-1 and MuRF1, suggesting that the decreased sarcomeric protein content was due to increased protein degradation by the proteasome system. The increase in atrogene expression could be inhibited by treatment with ActR2B-Fc or follistatin. The same authors also showed that myostatin acts through Smad3 to influence the expression of FOXO1 and atrogin1 and thus regulate proteolysis. Interestingly, the expression of MuRF1 and FOXO3 wasn't altered by Smad3 inhibition and the authors argued for a role of Smad2 in regulating these proteins. It may be that the effect of myostatin on protein degradation is dependent on the experimental model used.

The role of myostatin on autophagy is a new and interesting area of investigation. Autophagy is a fundamental cellular process that degrades old and malfunctioning organelles which are later replaced by newly synthesised ones. Myostatin and TGF-β were shown to induce autophagy in C2C12 cells. Myostatin induced the formation of autophagosomes and the expression of autophagy-related genes, e.g. ATG-4B and ULK-2. (Lee et al. 2011) Interestingly, autophagy is required for maintenance of skeletal muscle mass. Atg7-null (a paramount gene in the autophagy pathway) mice presented decreased muscle mass, absolute and specific force, misaligned Z-lines, and abnormal mitochondria and membrane structures. (Masiero et al. 2009) Similarly, mice lacking histone deacetylases (HDAC) 1 and 2 specifically in skeletal muscle also present abnormalities such as mitochondrial dysfunction and sarcomere degeneration

and perinatal lethality (Moresi et al. 2012). Interestingly, some of the beneficial effects of exercise training on skeletal muscle seem to be autophagy-related (He et al. 2012; Matsakas et al. 2012). Lee et al. (2011) and Mendias et al. (2011) suggested that myostatin blocking results in accumulation of misfolded proteins. The accumulation of misfolded and thus non-contractile proteins could also contribute to decreased specific force.

4. THE EFFECTS OF MYOSTATIN DEFICIENCY OR BLOCKING

Myostatin blocking has been studied for the last decade as a potential therapeutic intervention for the treatment of muscle-wasting conditions. Various approaches have been used to block myostatin signalling, e.g. myostatin antibodies (Bogdanovich et al. 2008), overexpression of the pro-peptide (Bogdanovich et al. 2005) or follistatin (Gilson et al. 2009), liver-targeted expression of a dominant negative myostatin (Morine et al. 2010a) and exon-skipping (Welle et al. 2011). These interventions, as well as naturally occurring mutations, resulted in increased skeletal muscle mass. Read more from an excellent review (Amthor & Hoogaars 2012).

Myostatin blocking has different mechanisms of action depending on the time of intervention, i.e. during embryonic phase or post-natal. Embryonic myostatin KO, as in the case of the natural mutations, elicits increased muscle mass due to both hyperplasia and hypertrophy. This shows that during embryonic development myostatin has a role in controlling skeletal muscle fibre number. However, post-natal myostatin deficiency results in fibre hypertrophy but not hyperplasia. (Lee 2004) Myostatin KO also interferes with fibre-type distribution. Myostatin KO animals have a higher proportion of fast fibres compared to wild-type animals or post-natal myostatin blocking. In accordance they also present higher levels of glycolytic enzymes and lower levels of oxidative ones. Myostatin KO embryos have different morphological characteristics than wild-type embryos. In mice, during the first myogenic wave myostatin KO embryos present a higher proportion of type 2 fibres with no difference in fibre number. Also, during the second myogenic wave they have higher fibre number and a higher percentage of adult fibre number compared to wild-type counterparts. (Matsakas et al.

2010) This is consistent with the observation that myostatin KO mice have a higher proportion of type 2 fibres and higher fibre number. Blocking myostatin early in the lifespan also has effects on fibre number and type. Inhibition of myostatin in neonate mice resulted in increased muscle mass due to both hyperplasia and hypertrophy. Moreover, although soleus weight did not change there was a change in fibre-type distribution with a 10% increase in type 2B fibres. (Morine et al. 2010a) Amthor et al. (2007) also found a shift towards fast fibres in myostatin null EDL muscle. They also found decreased oxidative enzyme content and decreased contraction and relaxation times. (Amthor et al. 2007)

Despite the evidence that myostatin affects muscle precursor cells the role of SC in myostatin inhibition-induced hypertrophy is still controversial. Previous *in vitro* studies showed that myostatin inhibits myoblast proliferation (Thomas et al. 2000) and satellite cell activation and proliferation (McCroskery et al. 2003), and also in some in vivo models (Gilson et al. 2009). Despite that some newer studies strongly suggest that SC do not have a prominent role in the hypertrophic process induced by myostatin inhibition (Amthor et al. 2009; Wang & McPherron 2012). The results of Amthor et al. (2009) suggest that muscle hypertrophy induced by myostatin blocking does not require satellite cell activity. They found that the myonuclear domain (the ratio of cytosolic content to DNA content) increased after myostatin inhibition, suggesting that the hypertrophy was due to increased protein synthesis and transcriptional/translational capacity. In contrast, Gilson et al. (2009) used follistatin over-expression to block myostatin and induce muscle hypertrophy. They found that gamma radiation, which blocks the proliferative capacity of SC, reduced the degree of hypertrophy. One criticism to this method is that gamma radiation can affect other cell types and thus interfere with processes in non-SC cells. Another methodological concern is that electroporation itself may cause some cellular damage and thus induce SC activation and proliferation. Rodriguez et al. (2011) reported no difference in nuclei per myotube between wild-type and myostatin KO cells, despite an 88% difference in myotube volume. Recently, Wang and McPherron (2012) showed that myostatin inhibition induces hypertrophy prior to SC activation. ActR2B-Fc treatment did not alter the SC pool. Additionally, the number of SC per myofibre was not different between groups despite increases in muscle mass and fibre CSA with ActR2B-Fc treatment. On the

other hand they found that the number of centrally located nuclei increased with ageing in myostatin KO animals compared to wild-type. The evidence so far suggests that myostatin inhibition is capable of inducing skeletal muscle hypertrophy regardless of the participation of SC. This idea is supported by a recent study from Se-Jin Lee's group (Lee et al. 2012). It is possible that SC need some physical/chemical stimuli, such as stretching or resistance exercise, to participate in muscle hypertrophy, and these stimuli are not provided by myostatin inhibition alone. A similar situation occurred with IGF-1. The latter acted as a hypertrophy agent only when a growth stimulus was present. The muscles of adult healthy mice did not respond to IGF-1 over-expression. Increased muscle mass and IGF-1 signalling was observed only in the muscles of mdx mice, and in healthy mice as a result of post-natal development. (Shavlakadze et al. 2009)

As with most therapeutic interventions myostatin deficiency is not without undesired effects. These include reduced capillary density, mitochondrial content and oxidative capacity of muscle (Baligand et al. 2010; Matsakas et al. 2012; Ploquin et al. 2012) . Also, the tendons of myostatin KO animals are hypocellular and brittle (Mendias et al. 2008). Accordingly, myostatin was shown to control fibrotic tissue formation and fibroblast proliferation (Li et al. 2008). So while myostatin negatively regulates muscle mass it also promotes fibrosis. Therefore, inhibiting myostatin could be doubly beneficial in a clinical context if these same changes were to occur when blocking myostatin in adults.

Some studies indicate that the muscles of myostatin KO animals have lower specific force than their wild-type counterparts (Amthor et al. 2007; Mendias et al. 2011). It is possible that the aforementioned tendon alterations interfere with force transduction, thus leading to decreased specific force. Likewise, myostatin-induced alterations of the extracellular matrix and connective tissue may also alter force transduction. On the other hand, some studies found no difference in specific force between wild-type muscle and myostatin-null muscle (Bogdanovich et al. 2008; Baligand et al. 2010). Mendias et al. (2011) found no difference in single fibre maximum isometric force between wild-type and myostatin-null muscle. They further argue that the greater force deficit after eccentric contractions in myostatin-null muscle is due to alterations in the connective tissue surrounding the muscle fibres. Focal adhesions are known to connect the extracellular matrix to the cytoskeleton and are thus an important component in

mechanotransduction. They have also been shown to influence insulin sensitivity and insulin/PI3K/Akt signalling (Goel et al. 2002; Huang et al. 2006; Bisht et al. 2007; Gupta & Dey 2009). Furthermore, focal adhesion kinase (FAK) have been shown to interact with TGF-β. TGF-β-induced apoptosis of chicken SC is associated with reduced activation of FAK (Li et al. 2009). The dy mice are an animal model of merosin-deficient congenital muscular dystrophy. Sakuma et al. (2004) showed that these mice present reduced levels of FAK at 12 weeks of age, whilst myostatin levels are increased compared to wild-type mice. Hong et al. (2011) showed that FAK is required for non-Smad TGF-β signalling in MEF cells. Although FAK phosphorylation was not required for TGF-β non-Smad signalling, it acted as a scaffold to couple TGF-β and PI3K signalling in a cell type-specific manner – in this case MEF cells. Myostatin inhibition may alter FAK signalling and the interaction between focal adhesions and the extracellular matrix. FAK activation could induce muscle hypertrophy whilst impaired connection of focal adhesions to the extracellular matrix could reduce muscle specific force and its adaptation to external loading.

The combination of myostatin inhibition and exercise intervention requires further studies. Most of the undesirable effects of myostatin inhibition could be counteracted by exercise. For example, Matsakas et al. (2012) showed that exercise (swimming and wheel running) increased muscle force, capillary density and oxidative capacity in myostatin KO muscle (Matsakas et al. 2012).

4.1 Activin receptor 2B as a blocking agent

ActR2B is one of the latest approaches to blocking myostatin signalling. It binds both activin A and myostatin and induces the same degree of hypertrophy as myostatin inhibition. The lack of an additive effect of inhibiting both myostatin and activin A suggests that these two factors have, at least partially, redundant effects on muscle mass. Over-expression of activin A was previously shown to induce muscle atrophy and inhibit muscle differentiation (Link & Nishi 1997; He et al. 2005; Gilson et al. 2009). It is noteworthy that the double-muscling phenotype of myostatin KO animals can be further enhanced by over-expression of follistatin, which also binds activin A (Gilson et al. 2009). Trendelenburg et al. (2012) presented detailed evidence that activin A

mediates interleukin 1- (IL-1) and tumour necrosis factor-α (TNF-α)-induced inhibition of skeletal muscle cell differentiation. They have shown that TAK-1, p38 and NF-κB link IL-1 and TNF-α signalling to activin A expression. IL-1- and TNF-α-dependent inhibition of differentiation rely on *de novo* secretion of activin A and subsequent activation of Smad signalling (Trendelenburg et al. 2012).

The therapeutic potential of ActR2B-Fc has been investigated and showed promising results. It increased muscle mass in both wild-type and mdx mice (Lee et al. 2005; Hoogaars et al. 2012) and prolonged survival in cancer cachexia conditions (Zhou et al. 2010). The increase in muscle mass and volume was also observed in healthy human subjects (Attie et al. 2012). Hoogaars et al. (2012) investigated the combination of ActR2B administration and exon skipping on wild-type and mdx mice. This combination had synergistic effects on skeletal muscle, i.e. while ActR2B increased muscle mass, exon skipping improved muscle function (specific and tetanic force and resistance to eccentric contractions). However, the response may differ between wild-type and mdx mice.

4.2 Myostatin antibodies, follistatin and genetic approaches

Antibodies against myostatin can also be used to block its activity. Bogdanovich et al (2008) have used this approach to inhibit myostatin in a murine model of limb-girdle muscle dystrophy 2C. They found increased fibre size, muscle mass and absolute strength. However, there were no improvements in histological paramters. In a human trial, Krivickas et al. (2009) investigated single fibre parameters in 6 patients with muscular dystrophy after treatment with a myostatin-neutralizing antibody. Type I fibres showed the most improvements; however, the small sample size (in terms of subjects and non-type I fibres) precluded more generalised conclusions. Furthermore, the patients had different types of muscular dystrophies and only one subject was used as control. In another human trial, Wagner et al. (2008) investigated the effects of a recombinant human antibody in patients with muscular dystrophies. They did not find any improvements in muscle mass, strength or histological parameters. Additionally, most of the patients presented adverse effects, especially at higher doses.

Follistatin is an endogenous protein that, similarly to ActR2B, binds both myostatin and

activin A. Follistatin has been previously used to inhibit myostatin signalling and induce muscle hypertrophy. To investigate the mechanisms behind follistatin-induced hypertrophy, Gilson et al. (2009) over-expressed follistatin in skeletal muscle of rats (both wild-type and myostatin-KO). Follistatin was able to induce muscle hypertrophy in both animal strains, which suggests that activin A also has a role in the control of muscle mass. To further support this idea they have over-expressed a mutant follistatin peptide which is able to bind myostatin but not activin A. The mutant peptide induced a lower degree of hypertrophy (compared to the wild-type peptide) in wild-type animals, but not in myostatin-KO animals. To address the role of SC in follistatin-induced hypertrophy the same authors used γ-radiation with the intent of disrupting the proliferative capacity of those cells. After radiation exposure over-expression of follistatin was still able to induce muscle hypertrophy, albeit to a lesser degree. Some criticism could be directed to the methods used by Gilson et al. (2009). The electroporation procedure itself could induce muscle regeneration. Given that the procedure creates small ruptures in the sarcolemma, these ruptures could be enough to trigger muscle regeneration and thus SC activation. Also, the radiation treatment could affect cells other than SC and interfere with biologic processes such as protein synthesis. Another study that investigated the mechanisms behind follistatin-induced muscle hypertrophy was done by Winbanks et al. (2012). They found that over-expression of follistatin in skeletal muscle increased muscle mass, strength, fibre diameter and fractional protein synthesis rate in wild-type animals. The signalling responses of myostatin-KO animals to follistatin over-expression were similar to their wild-type counterparts. The phosphorylation of Akt, TSC2, mTOR, S6K, rpS6 and 4EBP1 also increased in response to follistatin over-expression. Furthermore, they found that Smad3 is the main mediator of follistatin's effects in skeletal muscle. Expression of a constitutively active Smad3 significantly reduced the aforementioned responses to follistatin over-expression. Interestingly, those responses were still observed independent of concomitant myostatin over-expression. The authors argued for a role of activin A (and possibly other TGF-β members) in controlling muscle mass.

Recently a study by Roberts et al. (2012) has used the genetic approach of transcriptional gene silencing (TGS) to inhibit myostatin activity. They used a siRNA against the myostatin promoter to reduce its protein levels. Furthermore, the

combination of TGS and post-transcriptional gene silencing (i.e. the use of interfering RNAs that target the mature RNA) yielded the most robust knock-down.

5. PURPOSES AND HYPOTHESES

The purposes of this study were:

1. To assess the effect of different doses and frequencies of (ActR2B) injection on skeletal muscle mass.

Hypothesis: no previous study has assessed the effects of different doses on muscle mass. We expect that twice a week injections will have a more pronounced effect on muscle mass. Wang and McPherron (2012) used 3 different doses (5, 10 and 20 mg/kg) for 2 weeks and found that only the highest dose had a significant effect on muscle mass. However, they used only once a week injections. We hypothesise that there will be some significant interaction between the different doses and frequencies used in this study.

2. To assess if muscle mass would increase disproportionately to capillary count.

Hypothesis: previous studies have shown that lack of myostatin can compromise muscle oxidative metabolism (Matsakas et al. 2012; Ploquin et al. 2012). Therefore we expect that capillary count will not increase in proportion to muscle mass.

3. To investigate the signalling pathways underlying the observed outcomes.

Hypothesis: in accordance with previous literature we expect to find increased mTOR activity (Morissette et al. 2009; Trendelenburg et al. 2009; Welle et al. 2009; Rodriguez et al. 2011). We also expect decreased MAPK signalling as some of its members were shown to mediate myostatin effects in skeletal muscle (Huang et al. 2007; Bish et al. 2010).

6. METHODS

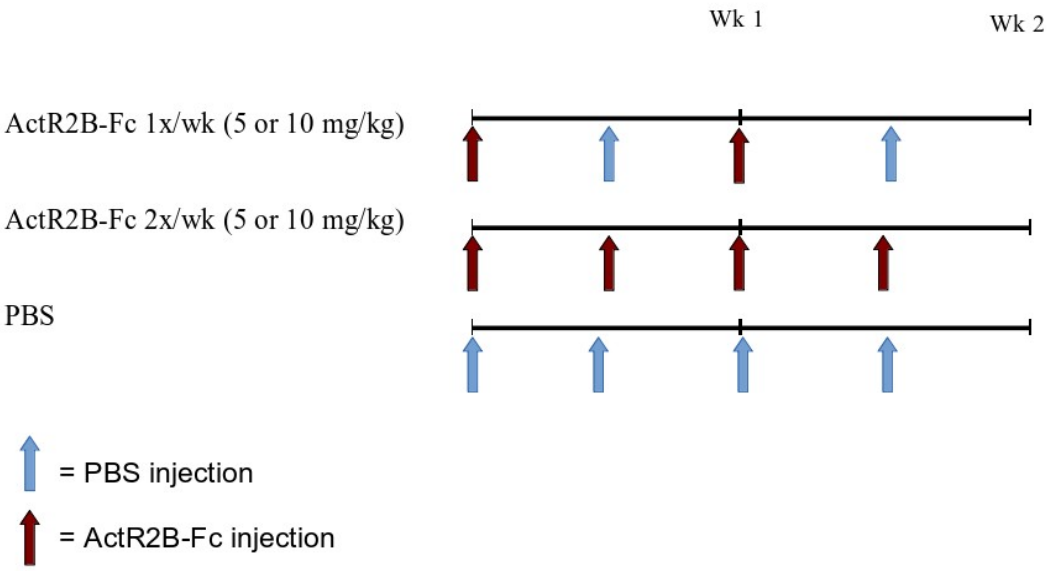
6.1 Animals

Male mice were 6-7 weeks old C57BL/10SnJ, all from The Jackson Laboratory (Boston, USA). They were housed in standard conditions (temperature 22°C, light from 8:00 AM to 8:00 PM). The mice had free access to tap water and food pellets (R36, 4% fat, 55.7% carbohydrate, 18.5% protein, 3 kcal/g, Labfor, Stockholm Sweden). The treatment of the animals was in strict accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. The protocol was approved by the National Animal Experiment Board (Permit Number: ESLH-2009-08528/Ym-23). All efforts were made to minimize suffering.

6.2 Experimental Design

ActR2B-Fc (5/10 mg/kg in PBS) was injected intraperitoneally to mice once or twice a week in experiment 1, and only a single injection in experiment 2. Dosing of the ActR2B-Fc was done from day one.

During the experiments all conditions were standardised. The mice were sacrificed after the experiments by cervical dislocation.



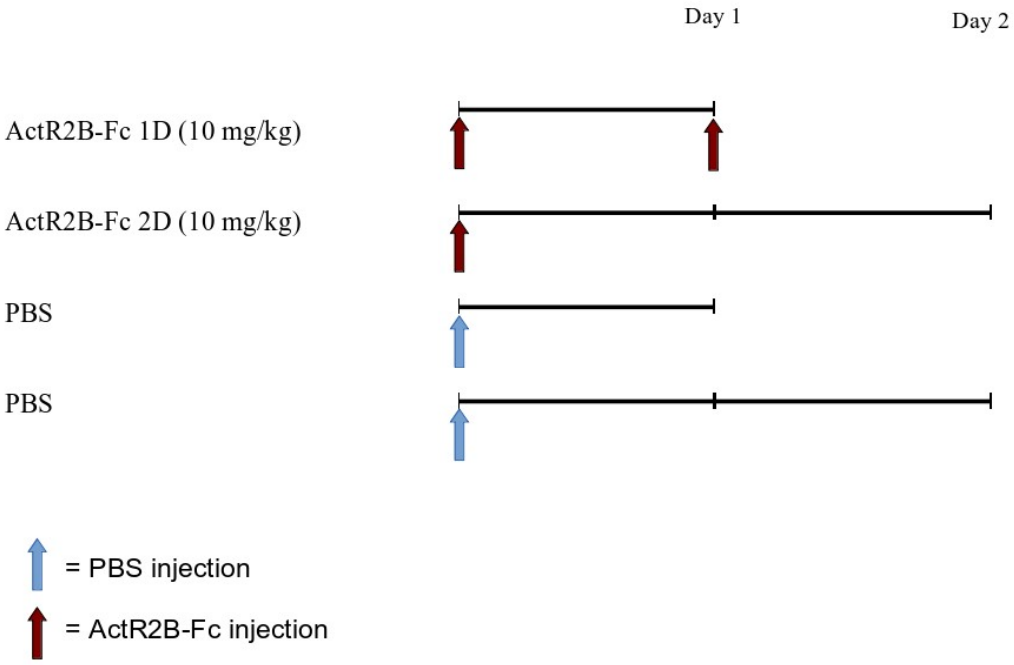


FIGURE 3. Experimental design of the 2-week (upper panel) and 2-day (lower panel) experiments.

6.3 ActR2B-Fc Production

The recombinant fusion protein was produced *in house* at the University of Helsinki by Olli Ritvos and Arja Pasternack. The ectodomain of human ActR2B was amplified via PCR with the following primers: GGACTAGTAACATGACGGCGCCCTGG and CCAGATCTGCGGTGGGGGCTGTCGG from a plasmid containing the human ActR2B sequence (in pCR-Blunt II-TOPO AM2-G17 ActR2B, I.M.A.G.E. clone #40005760, The IMAGE Consortium). A human IgG1 Fc domain with a C-terminal His6 tag was produced by PCR amplifying the Fc domain (GCAGATCTAATCGAAGGTCGTGGTGATCCCAAATCTTGTGAC and TCCCTGTCTCCGGGTAAACACCATCACCATCACCATTGAGCGGCCGCTT) from the pIgPlus expression plasmid. The subcloning of these products was done into the pGEM-T easy (Promega) vectors, sequenced, and fused before cloning into the expression vector pEFIRES-p. For the final production of the chimeric protein, chinese hamster ovary (CHO) cells were transfected with the above mentioned ActR2Becd–FcHis6 expression vector via lipofection (Fugene 6, Roche) and selected

with puromycin (Sigma–Aldrich). During expansion, cells were grown in DMEM supplemented with 2 mmol/l L-glutamine, 100 ul/ml streptomycin, 100 IU/ml penicillin and 10% FCS. DMEM–Hams F-12 (1:1) supplemented with 2 mmol/l L-glutamine, 100 ul/ml streptomycin, 100 IU/ml penicillin, 0.01% BSA and Heparin was used at 6.6 ml/liter and the heparin preparation was Fragmin 2500 IE/ml (Pfizer). Cell culture supernatants were clarified by filtration through 0.22 μm membrane (Steritop, Millipore). NaCl, 1 M, and imidazole, 5 mM, were added into the clarified supernatants, and the solution was pumped through a Ni2+-loaded HiTrap column (GE Healthcare Life Sciences, Uppsala, Sweden) at 4°C. Protein was eluted with raising imidazole concentrations, dialyzed against PBS and finally concentrated with Amicon Ultra concentrator (30 000 MWCO, Millipore).

Importantly, our ActR2B-Fc was shown to effectively inhibit myostatin, activin A and activin B signalling in an *in vitro* bioassay (unpublished results by Ritvos and Pasternack).

6.4 Muscle Sampling

The mice were sacrificed by cervical dislocation. Lower leg muscles soleus, gastrocnemius, MQF, extensor digitorum longus and tibialis anterior were immediately removed, weighed, and frozen in liquid nitrogen. Muscle weights reported are always average weights of left and right legs. The proximal part of of gastrocnemius muscle was mounted on an O.C.T. embedding medium (Tissue Tek, Sakura Finetek Europe) orientating muscle fibres vertically and snap frozen in isopentane cooled with liquid nitrogen.

6.4.1 Muscle Protein Synthesis: in vivo SUnSET

For muscle protein synthesis analysis we used a method developed by P. Pierre (Schmidt et al. 2009). This non-radioactive technique known as surface sensing of translation (SUnSET) was developed and validated against radioactive-based methods for measuring changes in protein synthesis in cell cultures and later validated in vivo in mice in different hypertrophy and atrophy settings by Hornberger's group (Goodman et al. 2011b). In this technique an injected antibiotic (puromycin – a structural analogue of tyrosyl-tRNA), when used at low concentrations is detected by a puromycin specific

antibody. The accumulation of puromycin-conjugated peptides into nascent peptide chains reflects well the rate of protein synthesis in many different in vitro and in vivo conditions.

For the in vivo measurements of protein synthesis mice were anaesthetized 1 or 2 days after ActR2B-Fc or PBS injection with inhalation anesthesia using isoflurane (MSS vaporizer with Fluovac absorbing unit, Harvard Apparatus). After that, mice were intraperitoneally injected of 0.040 μmol/g puromycin (Calbiochem, Darmstadt, Germany) dissolved in 200 μl of PBS. At exactly 25 min after injection of puromycin, mice were euthanized by cervical dislocation. Tissue collection timing was carefully monitored: gastrocnemius muscle was isolated 30 minutes (± 0.5 min.) after puromycin injection and then frozen in liquid nitrogen. The exact time point is important as it was shown in a pilot experiment that our puromycin assay was time-sensitive for incorporation; i.e. the longer the assay the more puromycin is incorporated into proteins and the higher the intensity of bands in western blots (data not shown).

6.4.2 Tissue Processing for the Protein Analyses

The whole calf muscle complex was pulverized in liquid nitrogen and then homogenized in ice-cold buffer as earlier (Hulmi et al. 2009; Hulmi et al. 2010): (20mM HEPES (pH 7.4), 1mM EDTA, 5mM EGTA, 10mM MgCl2, 100mM, b-glycerophosphate, 1mM Na3VO4, 2mM DTT, NP-40 1%, 0.2% sodium deoxycholate, and 3% protease and phosphatase inhibitor coctail (P 78443; Pierce, Rockford, USA). Muscle homogenates were rotated for 30 min at 4°C. Then one part for the protein puromycin incorporation examination with western blotting, the sample was centrifuged only 500 g 5 minutes to get away cell debris. For the signalling analysis the other part of the sample was centrifuged at 10 000 *g* for 10 min at 4°C. Total protein content was determined using the bicinchroninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, USA) in triplicates with an automated KoneLab device (Thermo Scientific, Vantaa, Finland).

6.4.3 Western Immunoblot Analyses

Aliquots of muscle lysate were solubilised in Laemmli sample buffer and heated at 95 °C to denature proteins. Samples containing 30 μg of total protein were separated by

SDS-PAGE for 40 min at 275 V using 4–20 % gradient or 10 % gels on Criterion electrophoresis cell (Bio-Rad Laboratories, Richmond, CA). Proteins were transferred to PVDF membranes at 300 mA constant current for 3h on ice at 4°C. Membranes were blocked in TBS with 0.1% Tween 20 (TBS-T) containing 5% fat-free dry milk for 2h and then incubated overnight at 4°C with primary antibodies. Membranes were then washed in TBS-T, incubated with secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG; Jackson ImmunoResearch Europe Ltd) for 1h followed by washing in TBS-T. All the antibodies were diluted in TBS-T containing 2.5% fat-free dry milk. Proteins were visualized by ECL (SuperSignal west femto maximum sensitivity substrate, Pierce Biotechnology, Rockford, USA) and quantified (band intensity x volume) using a ChemiDoc XRS in combination with Quantity One software (version 4.6.3. Bio-Rad Laboratories, USA).

For the protein puromycin incorporation the samples were incubated overnight at 4 °C with mouse IgG2a monoclonal anti-puromycin antibody (clone 12D10, 1:5000) dissolved in TBST containing 2% BSA. The automatic band detection of the Biorad software was used to analyse intensities of all the visible bands of both puromycin incorporated proteins as well as ubiquitinated proteins (see below). The sum of all the bands was taken into analysis. The result was confirmed by using different sensitivities of band recognition or quantitating the intensity of the whole lane (data not shown).

The uniformity of protein loading was confirmed by staining the membrane always with Ponceau S and by re-probing the membrane with an antibody against GAPDH (Abcam, Cambridge, USA). The results are presented as relative to Ponceau S at ~42 kDa. The mean results remained unchanged when presented as unrelated or relative to GAPDH (not shown).

Antibodies. Antibodies recognized phosphorylated rpS6 at $Ser^{240/244}$, ERK 1/2 MAPK at Thr^{202}/Tyr^{204} (Cell Signaling Technology, Beverly, MA). Moreover, total proteins of rpS6 and ERK 1/2 were analysed using specific antibodies (Cell Signaling Technology) by reprobing the membrane after respective phospho-antibodies.

6.4.4 Muscle Immunohistochemistry

Gastrocnemius muscle cross-sections cut with cryomicrotome were stained for membrane (antibody against caveolin 3 (ab2912, Abcam, UK), dilution 1:100) and

capillaries (isolectin, Invitrogen). Image analysis of fibre CSA and capillary density were performed with specific software (ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA). The number of muscle fibres analysed per sample ranged from 300 to 500.

6.5 Statistical Methods

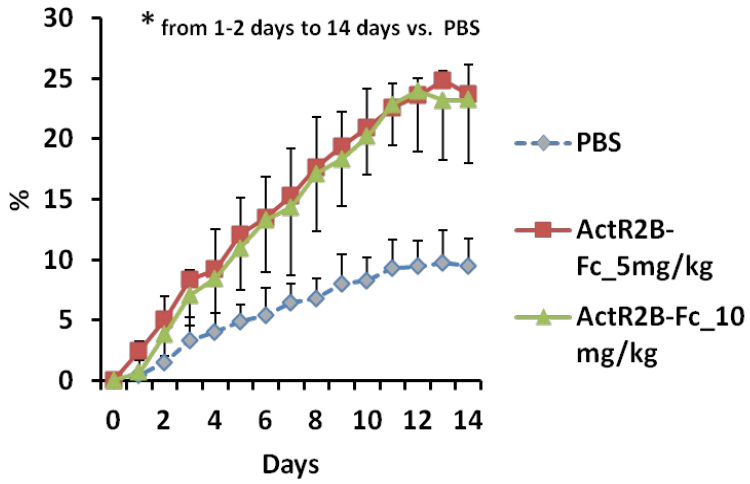
Differences between groups were evaluated by unpaired t-test or a two-way analysis of variance where appropriate. When the Shapiro-Wilk test revealed that western blot data was not normally distributed, all those values were log-transformed. LibreOffice Calc (The Document Foundation, Germany) and SPSS version 13.0 for Windows was used for statistical analyses (SPSS, Inc., Chicago, IL). The level of significance was set at $P \leq 0.05$. Data are expressed as means ± SD, except where designated.

7. RESULTS

7.1 Outcome Results

Body mass was significantly ($p < 0.05$) higher (compared to PBS) in response to ActR2B-Fc injection from day 2 and remained significantly higher throughout the 2-week period (figure 3). We found no difference between doses or frequency of injection. Therefore, the once and twice a week groups were pooled for further analyses.

A



B

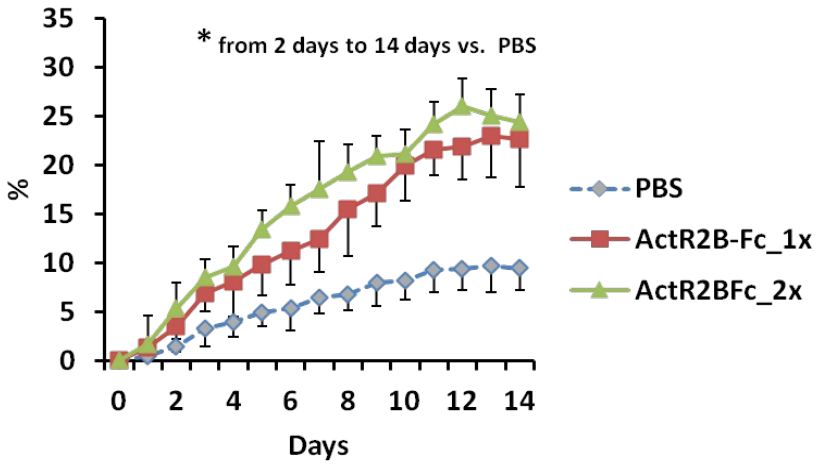


FIGURE 4. Percent change in body weight. A: PBS (blue square), ActR2B-Fc 5 mg/kg (red square) and ActR2B-Fc 10 mg/kg (green triangle). * $p < 0.05$ vs. PBS from day 2. B: PBS (blue square), ActR2B-Fc once a week (red square) and ActR2B-Fc twice a week (green triangle). * $p < 0.05$ vs. PBS from day 2.

Muscle masses were significantly higher ($p < 0.05$) in all ActR2B-Fc groups compared

to PBS (table 1 and figure 4). Once again there was no difference between 5 and 10 mg/kg. Quadriceps femoris (MQF) responded in a frequency-dependent manner ($p < 0.05$), whilst the other muscles showed no statistical difference due to dose or frequency of injection.

TABLE 1. Muscle weights

	PBS	ActR2B-Fc1x	ActR2B-Fc2x
Soleus (mg)	7.03 ± 0.38	7.93 ± 0.68	8.48 ± 0.14
EDL (mg)	9.30 ± 0.76	11.46 ± 1.34	11.33 ± 1.05
TA (mg)	36.33 ± 3.52	45.75 ± 2.36	48.22 ± 3.42
Gastrocnemius (mg)	128.09 ± 7.88	153.65 ± 10	162.52 ± 9.39
MQF (mg)	175.64 ± 10.56	211.65 ± 4.85	225.85 ± 14.17

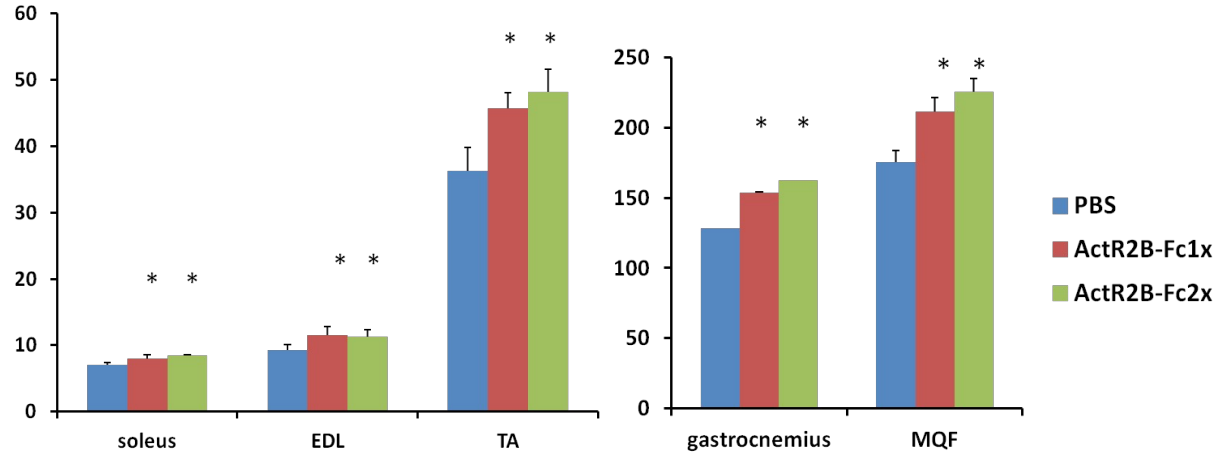


FIGURE 5. Weights of soleus, extensor digitorum longus (EDL) and tibialis anterior (TA) (left) and gastrocnemius and quadriceps femoris (MQF) (right). * $p < 0.05$ vs. PBS.

Fibre CSA increased with ActR2B-Fc treatment, with no difference between once or twice a week injections (1059.97 ± 485.11, PBS; 1164.10 ± 543.51, sActR2B 1x/week; 1155.71 ± 681.89, sActR2B 2x/week).

Capillary density decreased in both ActR2B-Fc groups compared to PBS (PBS: 472.93 ± 93.9; ActR2B-Fc1x: 409.2 ± 80.53; ActR2B-Fc2x: 366.24 ± 78.46; $p < 0.05$. Figure 5 lower panel). There was a borderline difference between once and twice a week injections ($p = 0.0506$).

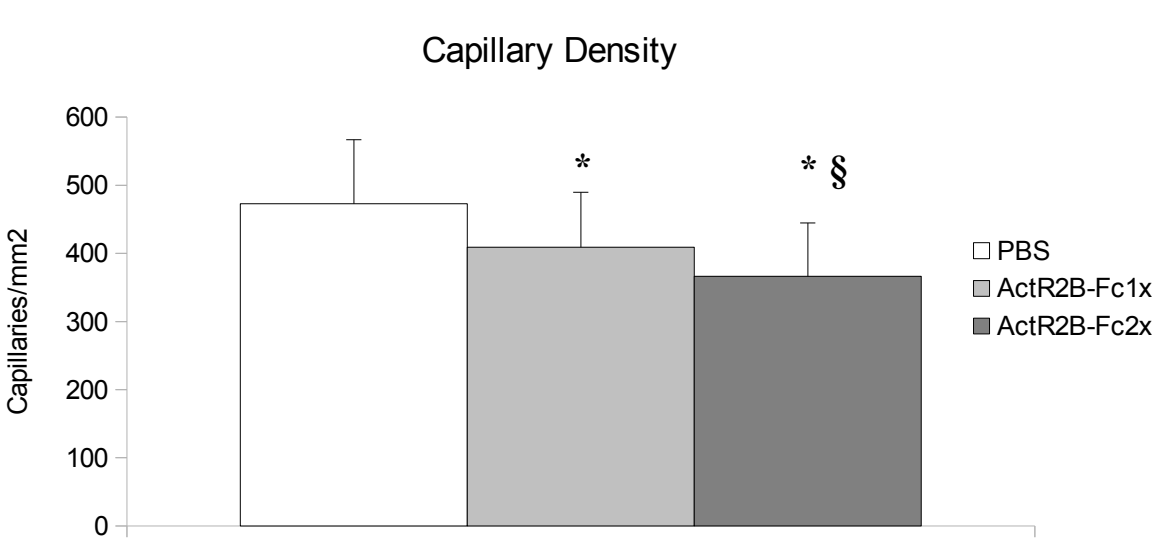
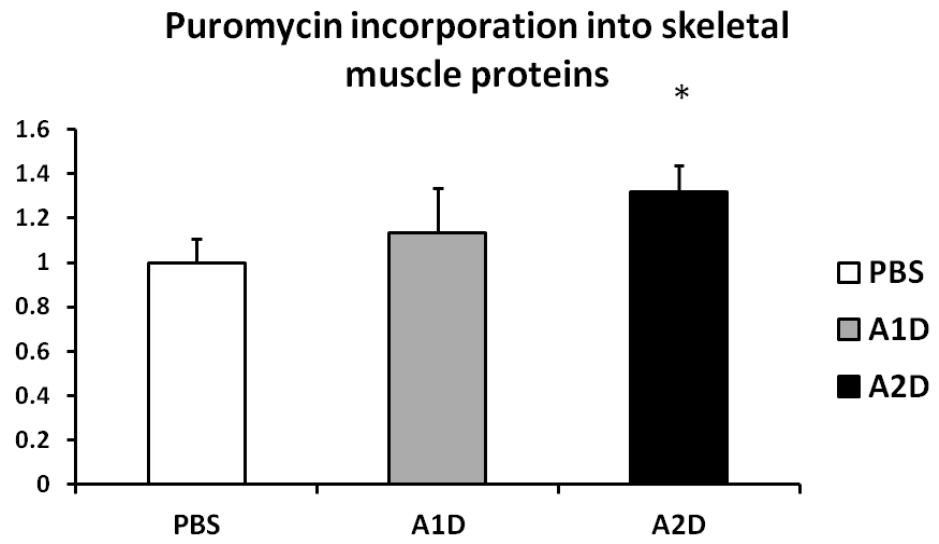


Fig 6. Fibre CSA distribution (upper), muscle capillary density (middle) and representative pictures (bottom) of fibre membrane (red) and capillaries (green). * $p < 0.05$ vs PBS, § $p < 0.05$ vs ActR2B-Fc1x.

7.2 Muscle Protein Synthesis and Signalling

To mechanistically examine the differences in muscle growth, we next assessed muscle protein synthesis. Muscle protein synthesis increased by ~30% 2 days after injection of a single dose of 10 mg/kg ActR2B-Fc (figure 6B). In accordance rpS6 phosphorylation (an indirect measure of mTORC1 activation) also increased compared to PBS 2 days post-injection (figure 6A). Knowing that ERK signalling is important in angiogenesis (Giuliani et al. 2004; Mavria et al. 2006) and mTOR activation (Wu et al. 2010; Winter et al. 2011), we then assessed the phosphorylation state of this MAPK member. Two days after a single injection of ActR2B ERK 1/2 phosphorylation decreased compared to PBS (figure 6C). Although a similar trend was observed in the 2x/week group (in which the last ActR2B-Fc injection was 4 days before sacrifice), it was not statistically significant. No changes in total content of ERK 1/2 or rpS6 were observed.

A


Puromycin incorporation into skeletal muscle proteins
*
1.6
1.4
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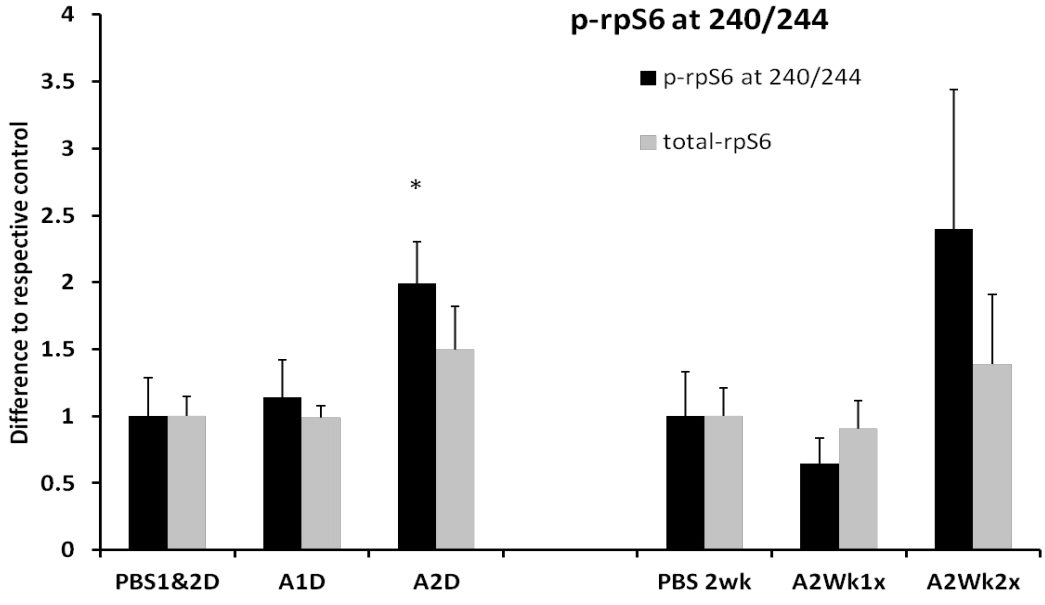
p-rpS6 at 240/244
p-rpS6 at 240/244
total-rpS6
Difference to respective control
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4
3.5
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2
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0.5
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PBS1&2D
A1D
A2D
PBS 2wk
A2Wk1x
A2Wk2x

C

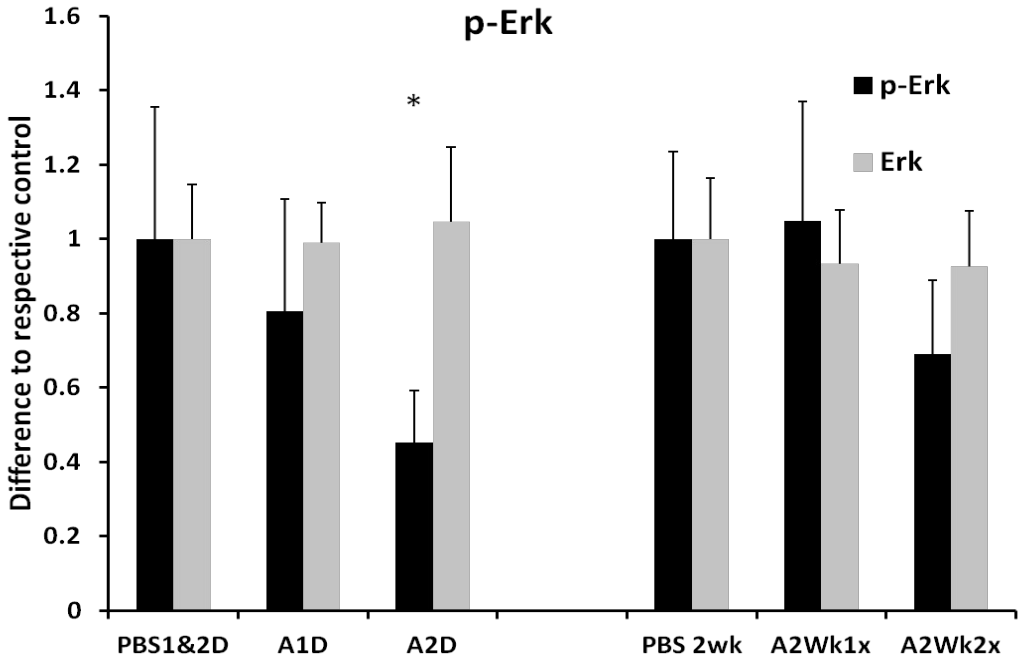

Figure 7. Protein synthesis measured by puromycin incorporation (A); phosphorylation status of rpS6 and ERK (B and C). * $p < 0.05$ vs PBS. A2Wk1x = ActR2B-Fc once a week; A2Wk2x = ActR2B-Fc twice a week; A1D = 1 day after 10 mg/kg ActR2B-Fc injection; A2D = 2 days after 10 mg/kg ActR2B-Fc injection.

8. DISCUSSION

The main findings of the present study were a) that our ActR2B-Fc is capable of stimulating skeletal muscle hypertrophy in healthy mice b) without significant differences between doses and frequency of injection, and c) that muscle capilarisation did not increase proportionately to muscle mass. These findings were related, at least acutely, to increased protein synthesis and mTOR activity (measured indirectly by rpS6 phosphorylation), while ERK 1/2 activity decreased.

In accordance with our hypothesis and previous studies that used ActR2B-Fc to block myostatin/activin signalling (Cadena et al. 2010; Morine et al. 2010b; Zhou et al. 2010; Hoogaars et al. 2012; Wang & McPherron 2012), our ActR2B-Fc treatment was capable of increasing body and muscle masses and fibre CSA. However, from the aforementioned studies only Cadena et al. (2010) and Wang and McPherron (2012) used healthy mice, similarly as here, to evaluate the effects of ActR2B-Fc administration. As far as we know this is the first study to assess the outcomes of different frequencies of injection (of ActR2B-Fc) on skeletal muscle mass and related signalling pathways. Contrary to our hypothesis, in the present study both body and muscle masses and fibre CSA increased with ActR2B-Fc treatment with no significant difference between doses and frequency of injection. MQF was the only muscle that presented a significant difference between frequencies of injection. It is possible that differences in fibre type distribution or receptor availability underlie this response. Also, the lack of major and more consistent differences between doses and frequencies may be due to receptor saturation, high affinity binding or a long protein half-life. Therefore, increasing the amount of circulating protein would have no additional effect on cell signalling. Recently, Wang and McPherron (2012) conducted a similar experiment using 3 different doses (5, 10 and 20 mg/kg). However, they have used only once a week injections. They found a trend for increased EDL weight with all the doses but only the 20 mg/kg was significantly different from control. All doses shifted fibre CSA distribution toward greater values. We found increased CSA with both 5 and 10 mg/kg doses, irrespective of injection frequency.

As hypothesised the number of capillaries per muscle area decreased with ActR2B-Fc treatment (in a dose-dependent manner). The degree of capillary density reduction was

similar to the increase in muscle mass. Therefore, we argue that the reduced capillary density was due to increased muscle mass only and there is no difference in the number of capillaries per fibre. Previously, myostatin-null animals did not differ from wild-type in the number of capillary contacts per fibre-type, with oxidative fibres (types I and IIa) having more contacts than glycolytic ones (type IIb). However, myostatin-null animals had a higher proportion of glycolytic fibres, which may result in a slight decrease in the number of capillaries per area. (Baligand et al. 2010) The reduction in capillary density may contribute to decreased muscle aerobic capacity and thus muscle function. Our unpublished results show decreased voluntary activity during the first 2 weeks (of a 7-week) ActR2B-Fc treatment. Coincidently, the largest increase in muscle mass also occurs during the first 2 weeks. Lack of adaptations such as increased capillarity and mitochondrial number/function that keep up with the increase in muscle mass may result in decreased central nervous system drive to perform physical activity. Even without a decrease in capillaries per fibre there may be metabolic consequences. If the oxygen supply is kept constant the larger fibre is likely to rely more on non-oxidative metabolism to meet its energy needs. If mitochondrial biogenesis does not follow the increase in muscle size the capacity for oxidative metabolism may be reduced. The results of Baligand et al. (2010) fit this scenario to a good degree. Blood oxygen extraction was delayed in myostatin-null animals during exercise and recovery, whilst mitochondrial oxidative phosphorylation was lower. The prolonged hyperaemia during recovery is probably a compensatory adaptation to ensure complete recovery, suggesting that blood vessel tone and blood flow regulation are intact in these animals.

The reduced capillary density in our study may be a result of decreased MAPK signalling. It was previously shown that ERK 1/2 and JNK influence angiogenesis. Milkiewicz et al. (2007) showed that JNK mediated MMP-2 production whilst ERK was necessary for VEGF production. (Milkiewicz et al. 2007) MMP allow the sprouting of endothelial cells through the basement membrane and interstitial space (Adams & Alitalo 2007). They are also involved in the processing of cytokines and growth-factors, including myostatin (Huet et al. 2001). ERK 1/2 signalling is involved in angiogenesis regulation in various cell lines (Giuliani et al. 2004; Pagès 2007). We found decreased phospho-ERK 2 days after a 10 mg/kg ActR2B-Fc injection, but not after 2 weeks of treatment. Previously, exercise intervention was capable of restoring capillary density

and muscle oxidative capacity in myostatin-null animals (Matsakas et al. 2012). Coincidently, ERK 1/2 phosphorylation has been shown to increase in an exercise intensity-dependent manner (Widegren et al. 2000), suggesting that ERK 1/2 may indeed be an important regulator of angiogenesis.

The role of MAPK in the context of myostatin inhibition is still dubious since MAPK may have both anabolic and catabolic effects in skeletal muscle (Haddad & Adams 2004; Li et al. 2005; Shi et al. 2009; Trendelenburg et al. 2009; Trendelenburg et al. 2012). ERK 1/2 inhibition prevented muscle wasting in tumour-bearing mice and rats. They found that ERK 1/2 up-regulation was mediated by TNF-α, a pro-inflammatory cytokine. ERK 1/2 inhibition also slightly reduced atrogin-1 expression in the gastrocnemius of tumour-bearing mice. Over-expression of follistatin did not alter ERK 1/2 levels, which led the authors to argue that ERK 1/2 activation is independent of myostatin. Furthermore, ERK 1/2 activation prevented SC proliferation and differentiation, whilst ERK 1/2 inhibition restored myogenin levels in tumour-bearing animals. (Penna et al. 2010) On the other hand, ERK 1/2 and Akt were shown to work in parallel to promote mTORC1 signalling. Both pathways acted distinctly to inhibit TSC2, thus leading to mTORC1 activation. Activation of both pathways presented an additive effect on mTORC1 activation, phosphorylating the latter in different residues. (Winter et al. 2011)

Protein synthesis increased ~30% 2 days after a single 10 mg/kg ActR2B-Fc injection. Accordingly, we also found increased phospho-rpS6 at the same time point, a marker of increased mTORC1 signalling, with no difference in total rpS6 content. After 2 weeks of treatment there was no difference in mTORC1 signalling among groups. This suggests that increased protein synthesis may indeed precede SC involvement, as argued by Wang and McPherron (2012). Prior to SC fusion with the pre-existing fibres a larger myonuclear domain (i.e. the ratio of cytoplasmic volume per DNA content) would require increased protein synthesis (per DNA content) for cellular growth, and based on our results this seems to be the case, even though SC content or activity was not investigated. Welle et al. (2009a) also reported increased phospho-rpS6 (albeit at different sites than ours) after myostatin antibody treatment, whilst the phosphorylation state of 4E-BP1 and Akt were not altered. We also found increased p-S6K1 and p-4EBP1 (not shown; these and other results are now published (Hulmi et al. 2012)).

Interestingly, rapamycin (an inhibitor of mTOR) treatment did not alter the increased protein synthesis rate induced by antibody treatment, although the phosphorylation of S6K and rpS6 were blunted (Welle et al. 2009a). It is possible that at least part of the myostatin/activin blocking effects on protein synthesis are accomplished through rapamycin insensitive mechanisms, and thus non-mTORC1 pathways.

One limitation to the present study is the short intervention time. Although the first two weeks is when the most prominent muscle hypertrophy occurs, other aspects of muscle physiology (such as synthesis of metabolic enzymes and connective tissue, angiogenesis and satellite cell activity) may have different time-courses. Perhaps during the period of more pronounced muscle growth the cellular machinery is excessively directed to protein synthesis and thus other aspects of cellular maintenance lag behind. Future studies should address those limitations, as well as the interaction between ActR2B-Fc administration and other factors know to influence skeletal muscle mass such as nutrition and physical activity. The combination of exercise and ActR2B-Fc treatment may be an optimal approach as exercise may attenuate the reduction in capillary density and oxidative metabolism in response to ActR2B-Fc treatment. Exercise also stimulates autophagy, which may prevent the synthesis of non-functional proteins and thus maintain proper muscle function.

In conclusion, the present study has shown that 2 weeks of ActR2B-Fc treatment is capable of increasing muscle and body weight, as well as fibre CSA. This increase is mediated, at least initially, by increased protein synthesis and mTOR signalling (as measured by rpS6 activity). Capillary density decreased in a dose-dependent manner, probably due to the degree of muscle mass increase and reduced activity of ERK 1/2. The acute changes in signalling were no longer observed after 2 weeks of treatment.

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