

MUSCLE HYPERTROPHY INDUCED BY MYOSTATIN INHIBITION IS SUPPRESSED BY
RAPAMYCIN ADMINISTRATION

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CHAPTER 1

LITERATURE REVIEW

Muscle is an important tissue for animal agriculture because as a main source of meat it provides high quality proteins, B vitamins, Fe and Zn to human, and improving the efficiency of meat production is crucial for continuous supply of this nutritious products to increasing human population. It appears that understanding the mechanisms involved in the regulation of muscle growth and development can contribute to developing strategies to improve skeletal growth. Recent studies have shown that the mTOR pathway plays an important role in muscle growth (Bodine et al., 2001; Pallafacchina et al., 2002). Recent studies have shown that myostatin is a potent negative regulator of skeletal muscle mass, and studies in lab animal studies have shown that Mstn suppression enhances skeletal muscle growth (Lee, 2001). It has also emerged that the mTOR pathway plays an important role in muscle growth (Bodine et al., 2001; Pallafacchina et al., 2002). Thus, in this literature review focuses on molecular and physiological aspects of the anabolic Akt/mTOR pathway and Mstn.

1.1 mTOR SIGNALING PATHWAY

Muscle is an important tissue for animal agriculture because as a main source of meat it provides high quality proteins, B vitamins, Fe and Zn to human, and improving the efficiency of meat production is crucial for continuous supply of this nutritious products to increasing human population. It appears that understanding the mechanisms involved in the regulation of muscle growth and development can contribute to developing strategies to improve skeletal growth. Recent studies have shown that the mTOR pathway plays an important role in muscle growth (Bodine et al., 2001; Pallafacchina et al., 2002).

The target of rapamycin (TOR) was first found in the budding yeast *Sacchomyces cervisiae* as a growth regulator, whose activity is suppressed by rapamycin, an antifungal agent discovered from *Streptomyces hygroscopicus* (Heitman et al., 1991). The mammalian counterpart of TOR (mTOR) was later found in mammalian cells as a 289 kDa serine/threonine kinase (Sabatini et al., 1994). The mTOR is also known as FKBP12-rapamycin associated protein (FRAP) or rapamycin and FKBP12 target (RAFT),

or rapamycin target (RAPT), and its activity is suppressed not directly by binding to rapamycin, but by rapamycin complexed with FKBP12 (FK506 binding protein). Studies have shown that mTOR signaling affects various cellular functions, thus playing an important role in cell proliferation, growth, and aging (Tobias et al., 2000). Many human diseases, including cancer, type 2 diabetes and neurodegeneration, have been shown to be associated with deregulation of the mTOR signaling (SG Dann et al., 2007).

1.1.1 Structure of mTOR complex

The mTOR protein is a 289 kDa serine/threonine kinase, which is a member of the evolutionally-conserved phosphoinositide 3-kinase (PI3K)-related kinase family. The mTOR interacts with several proteins to form two distinct multi-protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Guertin and Sabatini, 2007). The mTORC1 and mTORC2 have shared and unique protein components (Fig.1.1). Both complexes have the catalytic mTOR subunit, G β L (also known as mammalian lethal with sec-13 protein 8, mLST8), DEP domain containing mTOR interacting protein (DEPTOR), and the Tti1/Tel2 complex. However, raptor (regulatory-associated protein of mammalian target of rapamycin) and PRAS40 (proline-rich Akt substrate 40 kDa) are found only mTORC1. Raptor, as a scaffold protein, can link the mTOR kinase with mTORC1 substrate, promoting mTORC1 signaling. PRAS40 appears to act as both component and substrate of mTORC1 and act as an inhibitory subunit of the mTORC1 complex. It has been shown that phosphorylation of PRAS40 by Akt and by mTORC1 itself induces dissociation of PRAS40 from mTORC1, relieving an inhibitory constraint of PRAS40 on mTORC1 activity (Wiza et al., 2012). The unique components in mTORC2 are rictor (rapamycin-insensitive companion of mTOR), mSIN1 (mammalian stress activated protein kinase interacting protein), and protor 1/2 (also known as PRR5). Rictor and mSin1 help mTORC2 assembly and signaling (Frias et al., 2006; Jacinto et al., 2006). Rictor has been shown to interact with protor-1, but the physiological function of this relation is still unclear (Thedieck et al., 2007).

1.1.1.1. mTORC1 substrates and function

mTORC1 perceives extra- and intra-cellular signals to promote anabolic and to inhibit catabolic cellular processes. Several factors, like growth factors, nutrients, energy, and stress can affect protein synthesis, cell growth, cell proliferation, and cell metabolism (Laplante and Sabatini, 2012). Also, these factors can promote degradative processes that breakdown cell components into amino acids or other small molecules. TORC1 also can raise transcription level of ribosomal RNAs and protein levels in yeast and mammals. When raptor binds to the mTOR, it alters the downstream of mTOR signaling, including S6K1 (ribosomal S6 protein kinase 1) and 4E-BP1 (eukaryotic initiation factor 4 E-binding protein) to link them with mTOR kinase. mTOR/raptor complex directly phosphorylates S6K1 on its hydrophobic motif site (Thr389) and 4E-BP1 on multiple sites (Thr 37/46, Thr70, Ser65). Raptor mutation at the raptor N-terminal conserved domain interfere 4E-BP1 binding and mTORC1 mediated 4E-BP1 phosphorylation in vitro, thus underscoring the importance of the raptor and 4E-BP1 interaction for mTORC1 signaling. Protein synthesis, cell growth, cell proliferation, and cell metabolism represent evolutionarily conserved TORC1 functions. Inhibition of cell growth and proliferation by mTORC1 is not well understood yet, however they are known be related with protein synthesis. S6K1 and 4E-BP1, the most important substrate of mTORC1, regulate protein translation. S6K1 regulates the eIF3 translation initiation complex at the 5'-methylguanosine cap of mRNAs. Stimulation of mTORC1 causes phosphorylation of S6K1 (at Thr389), and recruits eIF3 complex, leading to increases in protein translation. Substrates of S6K1, such as ribosomal protein S6, eIF4B, eEF2K (eukaryotic elongation factor 2 kinase), PDCD4 (programmed cell death 4), CBP80 (cap-binding protein of 80 kDa), and SKAR (S6K1 Aly/ REF-like target), serve as an important role in translation control. mTORC1-mediated S6K1 phosphorylation and following S6K1 mediated phosphorylation of the 40S ribosomal protein S6 were considered to increase translation of 5' terminal oligopyrimidine mRNAs, which encode ribosomal proteins and translation elongation factors. Therefore, this translation prepares cells for high level of protein synthesis. 4E-BP1 plays as a translational suppressor, and this suppressor binds and inhibits eIF4E. Upon activation, mTORC1 phosphorylates 4E-BP1, which in turn increases 4E-BP1 dissociation from eIF4E, which helps to

participate in the formation of eIF4F complex along with eIF4G and eIF4A. The complex formation is required for the initiation of cap-dependent translation. mTORC1 also increases lipogenesis by promoting the transcription of peroxisome proliferation activated receptor and sterol regulatory element binding protein activities. Therefore, mTORC1 leads cell growth by increasing both protein and lipid synthesis.

1.1.1.2. mTORC2 substrates and function

mTORC2 consists of six different proteins including mTOR, rapamycin- insensitive companion of mTOR (Rictor), mammalian stress activated protein kinase interacting protein (mSIN1); protein observed with Rictor-1 (protor-1); mLST8; and DEPTOR. There is some evidence that Rictor and mSIN1 interacting each other, and forms the structural foundation of mTORC2 (Frias et al., 2006; Jacinto et al., 2006). Rictor also interacts with protor-1, but the physiological function of this relation is still unclear (Thedieck et al., 2007). Similar to its role in mTORC1, DEPTOR negatively controls mTORC2 activity (Peterson et al., 2009). Moreover, DEPTOR is considered as an endogenous inhibitor of mTORC2. mLST8 serves as an essential component for mTORC2 function, as knockout of this essential protein significantly inhibits the stability and activity of this complex (Guertin et al., 2006). Contrast to mTORC1, relatively little is known about role of mTORC2. However, many important studies have been made these days. Using different kinds of genetic methods, mTORC2 was proven to play important roles in various biological processes, including cell survival, metabolism, proliferation and cytoskeleton organization. Cell survival, metabolism and proliferation are highly relying on Akt activation, which increases the phosphorylation of various effectors (Manning and Cantley, 2007). Akt activation needs its phosphorylation at Ser308 by phosphoinositide dependent kinase 1 (PDK1), and at Ser473 by a kinase that was unclear for many years, but was proven to be mTORC2 in 2005 (Sarbasov et al., 2005). Several studies have demonstrated that ablation of various mTORC2 components specifically blocks Akt phosphorylation at Ser473 and the downstream phosphorylation of some Akt substrates, demonstrating that mTORC2 activates Akt by phosphorylation of Ser 473, a site required for maximal Akt activity (Guertin et al., 2006; Jacinto et al., 2006). Suppression of Akt following mTORC2 reduction cause the phosphorylation of the forkhead box protein O1 (FoxO1) and FoxO3a transcription factors, which

regulates the expression of genes including stress resistance, metabolism, cell-cycle arrest and apoptosis (Calnan and Brunet, 2008; Greer and Brunet, 2008). However, the phosphorylation of TSC2 and GSK3 is not affected by mTORC2 inhibition. A recent study shows that serum- and glucocorticoid-induced protein kinase 1 (SGK1), which shares homology with Akt, was regulated by mTORC2 (Garcia-Martinez and Alessi, 2008). Many studies proved that inhibition of mTORC2 affect actin polymerization and perturb cell morphology (Jacinto et al., 2004; Sarbassov et al., 2004). These studies suggested that mTORC2 regulates the actin cytoskeleton by promoting protein kinase Ca (PKCa) phosphorylation, phosphorylation of paxillin and its relocalization to focal adhesions, and the GTP loading of RhoA and Rac1. However, the molecular mechanism that the mTORC2 controls these processes is still not clear.

1.1.2. Upstream regulators of mTOR

mTOR integrates various signals to regulate cell growth. Four major upstream inputs have been implicated in TOR signaling: growth factors, nutrients, energy, and stress.

1.1.2.1. Growth factors

Growth factors, such as insulin and insulin like growth factors (IGF-1) activate mTORC1 via Ras signaling pathway. As the insulin binds to its cell surface receptor, level of the tyrosine kinase activity increases and recruit the insulin receptor substrate 1 and produce phosphatidylinositol (3,4,5)-triphosphate [PtdIns (3,4,5)P3] through the activation of PI3K, resulting in activation of Akt at the plasma membrane. The activation of Akt induces the phosphorylation of TSC2, leading to inactivation of TSC1/2 and consequent stimulation of mTORC1. Furthermore, stimulation by growth factors can activate mTORC1 by promoting the phosphorylation and dissociation of PRAS40 from mTORC1 in a TSC1/2 independent manner by increasing the phosphorylation and dissociation of PRAS40 from mTORC1 (Sancak et al., 2007; Vander Haar et al., 2007; Wang et al., 2007). mTORC1 stimulates S6K1 and increase the level of phosphorylation of IRS1 and suppresses its stability (Harrington et al., 2005). This regulation of pathway is characterized as S6K1 dependent negative feedback and it has been found that abnormal regulation of this pathway is responsible for both metabolic diseases and tumorigenesis (Manning, 2004).

1.1.2.2. Energy status

Cellular energy status is transferred to mTORC1 via AMP activated protein kinase (AMPK) that is a major sensor of intracellular energy condition (Hardie, 2007). Low level of energy states activates AMPK and phosphorylates TSC2, which stimulates the GAP activity of TSC2 towards Rheb and suppresses mTORC1 activation (Inoki et al., 2003). Moreover, AMPK suppresses mTORC1 in response to energy deficiency by directly phosphorylating Raptor (Gwinn et al., 2008).

1.1.2.3. Oxygen level

Level of oxygen states influences mTORC1 activation through many pathways (Wouters and Koritzinsky, 2008). In low level of oxygen conditions, decrease in ATP level stimulates AMPK, which increase TSC1/2 activation and suppresses mTORC1 signaling (Arsham et al., 2003; Liu et al., 2006). Low oxygen level can also stimulate TSC1/2 through transcriptional regulation of DNA damage response 1 (REDD1) (Brugarolas et al., 2004; Reiling and Hafen, 2004). REDD1 inactivates mTORC1 signaling by TSC2 releasing from its growth factor induced association with 14-3-3 proteins (DeYoung et al., 2008). Moreover, promyelocytic leukemia (PML) tumor suppressor and BCL2/adenovirus E1B 19 kDa protein interacting protein 3 (BNIP3) lower mTORC1 signaling in low oxygen level by disturbing the interaction between mTOR and its positive regulator Rheb (Bernardi et al., 2006; Li et al., 2007).

1.1.2.4. Amino acids

Amino acids serve as a strong signal that positively controls mTORC1 (Guertin and Sabatini, 2007). Recent studies show that leucine, an essential amino acid, activates mTORC1, and it is transported into cells in a glutamine-dependent manner. Glutamine, which is transported into cells through SLC1A5 [solute carrier family 1 (neutral amino acid transporter) member 5], import leucine through heterodimer system consist of SLC3A1 [solute carrier family (activators of dibasic and neutral amino acid transport) member 2]. The system by which intracellular amino acids transfer to mTORC1 is still unclear. The activation of mTORC1 by amino acid is independent of TSC1/2, because the mTORC1 pathway is sensitive to amino acid deficiency in cells that lack TSC1 or TSC2 (Nobukuni et al., 2005). Some studies

show that human vacuolar protein-sorting associate protein 34 (VPS34) is involved in nutrient sensing (Nobukuni et al., 2005); however, the exact role of human VPS34 is still unclear. Recent studies show that the Rag proteins, a family of four related small GTPases, interact with mTORC1 in an amino acid sensitive manner and are necessary for the activation of the mTORC1 pathway by amino acids (Kim et al., 2008; Sancak et al., 2008). In the presence of amino acids, Rag protein interacts with Raptor and boost relocalization of mTORC1 from different locations throughout the cytoplasm to a perinuclear site where its catalyst Rheb is located (Sancak et al., 2008). The physical dissociation of mTORC1 and Rheb under amino acid deficiency probably explains that growth factors and Rheb activators cannot stimulate mTORC1 pathway in the absence of amino acid.

1.1.3. Role of Akt/mTOR signaling network in the regulation of skeletal muscle growth

The role of the mTOR pathway in muscle growth was demonstrated by in vivo studies. The hypertrophic responses induced by overload or regenerating muscle growth are blocked by rapamycin (Pallafacchina et al., 2002). In addition, transgenic mice overexpressing TSC1 specifically in skeletal muscle showed a defect in muscle growth through down regulation of mTOR activation (Wan et al., 2006). It was also reported that muscle specific ablation of mTORC1 (by ablating raptor) results in dystrophic phenotype (Bentzinger et al., 2008). One of the major upstream regulators of Akt is phosphatidylinositol-3-OH kinase (PI3K), a lipid kinase mediating the action of IGF-1. It is well established that IGF-1 is a potent stimulator of skeletal muscle hypertrophy as demonstrated by an increase in muscle mass and strength by forced expression of IGF-1 in mice (Coleman et al., 1995). Studies have shown that the PI3K/Akt pathway plays an important role in mediating the hypertrophic action of IGF-1, as well as other anabolic stimuli, such as overloading, mechanical stimuli and insulin (Glass, 2005; Nader, 2005). Expression of DNA constructs encoding constitutively active forms of either PI3K or Akt induced muscle hypertrophy both in in vitro (Rommel et al., 1999) and in vivo experiment (Bodine et al., 2001; Pallafacchina et al., 2002). Furthermore, activation of the Akt/mTOR pathway could oppose muscle atrophy induced by disuse (Bodine et al., 2001).

mTORC1 activation by Akt induces the phosphorylation of two downstream effectors, ribosomal protein p70S6k and 4E-BP1, a suppressor of the cap binding protein eIF4E, resulting in increased protein translation (Sarbasov et al., 2005). Supporting evidences that the role of p70S6k as a downstream mediator for the mTOR anabolic pathway come from the p70S6k null mice, which has smaller muscle fiber size. The hypertrophic response of the p70S6k-null mice to IGF-1 and activation of Akt is blocked (Ohanna et al., 2005). Anabolic stimuli, such as overloading, resistance exercise, and essential amino acid ingestion, have been shown to increase the phosphorylation of p70S6k and 4E-BP1 along with a corresponding increase in protein synthesis (Sarbasov et al., 2005). Conversely, atrophying muscles have decreased level of phosphorylation of the downstream targets of mTORC1 (Bodine et al., 2001; Hornberger et al., 2001).

1.2. Myostatin, a negative regulator of skeletal muscle growth

Myostatin (Mstn), also known as a growth and differentiate factor-8 (GDF-8), is a member of the transforming growth factor-beta (TGF- β) superfamily and a negative regulator of skeletal muscle mass (McPherron et al., 1997; Lee and McPherron, 2001). Knock-out of the Mstn gene increased muscle mass size by 2-3 times in mice (McPherron et al., 1997). The increase in muscle weight of Mstn knock-out mice was caused by both muscle fiber hyperplasia and hypertrophy. Moreover, studies have shown that mutation of Mstn gene result in dramatic muscle mass increase in cattle (Kambadur et al., 1997; Grobet et al., 1998), dogs (Mosher et al., 2007), sheep (Clop et al., 2006) and humans (Schuelke et al., 2004).

1.2.1. Myostatin protein processing, activation and inhibition

Mstn, as a member of TGF- β superfamily proteins, shows biochemical similarity to the other member of the family. Mstn is translated as a precursor protein composed of a signal peptide, and N-terminal propeptide (prodomain) and a C-terminal mature/active domain (Zimmers et al., 2002). The signal peptide (24 amino acids) serves as a transporter of Mstn precursor protein into the endoplasmic reticulum, where it is eliminated by the proteolytic processing prior to secretion. cDNA sequencing analysis proved that the precursor Mstn have 375 AA in humans, baboons, cattle, pigs, sheep, turkeys and

chickens, but there are 376 AA in rodents (McPherron and Lee, 1997). Calcium-dependent serine protease called furin proteolytically cleaves precursor protein to the active form of Mstn containing 109 AA upon removal of the N-terminal prodomain at a conserved tetrabasic (RSRR) site (Lee and McPherron, 2001; Thies et al., 2001). A study by Jin et al. (2004) show that porcine Mstn propeptide is necessary for proper folding of active domain of Mstn. Mature Mstn is well conserved evolutionally with identical AA composition in human, mouse, rat, pig, chicken and turkey species and with only one AA difference in baboon, 2 AA differences in bovine and 3 AA difference in ovine species (McPherron and Lee, 1997). Similar to many other members of TGF- β superfamily, the mature form as well as the propeptide form of Mstn appears to form homodimers by disulfide-bonds (McPherron and Lee, 1997; Lee and McPherron, 2001; Jin et al., 2004). Mstn prodomain does not produce disulfide-linked homo dimers (Lee and McPherron, 2001; Jin et al., 2004). The mature Mstn homo-dimer is biologically active, and is capable of binding to ActRIIB receptors on skeletal muscle cells to exert its negative regulatory role on muscle cell (Lee and McPherron, 2001). After the proteolytic processing of the precursor, Mstn is secreted as an inactive latent complex of a mature dimer bound by two propeptides, thus the propeptide is functioning to suppress Mstn activity. In support of the role of propeptide to inhibit Mstn biological activity, the overexpression of Mstn propeptide in mice resulted in a dramatic increase in skeletal muscle mass (Lee and McPherron, 2001, Yang et al., 2001). In addition, the administration of recombinant Mstn propeptide (Li et al., 2010) or plasmid- or adeno-associated virus-mediated delivery of Mstn propeptide induced an increase in muscle mass (Matsakas and Patel, 2009; Hu et al., 2010).

Binding of mature Mstn to its receptor (ActRIIB) for induction of intracellular signaling cascade, thus, requires dissociation of the propeptide from the latent complex. The propeptide binding to the mature region can be physically or biochemically destabilized after it is cleaved by protease, such as bone morphogenetic protein-1 /tolloid (BMP-1/TLD) family of metalloproteinases (Wolfman et al., 2003; Lee, 2008). The importance of BMP-1/TLD metalloproteinase in regulating Mstn latency in vivo is supported by studies using a mutant form of the propeptide in which aspartate 76 was mutated to alanine (Wolfman et al., 2003, Li et al., 2010). The mutant form of propeptide resistant to cleavage by BMP-1/TLD

metalloproteinases caused a significant increase in muscle mass when injected into adult mice (Wolfman et al., 2003). Mice engineered to carry a germ line point mutation of propeptide that rendered to be resistant to the BMP-1/TLD metalloproteinases exhibited an increase in skeletal muscle mass (Lee, 2008), suggesting that the BMP-1/TLD metalloproteinases is involved in activating latent Mstn in vivo and that molecules capable of inhibiting these proteinases may be an effective therapeutic strategy for enhancing muscle growth in the clinical settings of muscle loss and degeneration.

Not only the propeptide, but also many other proteins also have the capability of binding and suppressing the activity of mature Mstn. Some studies showed that follistatin can function as a potent Mstn antagonist and serves an important role in controlling Mstn activity. Follistatin can block Mstn activity in both receptor binding and reporter gene assay in CHO cells (Zimmers et al., 2002).

Overexpression of follistatin in muscle caused dramatical increases in skeletal muscle growth in mice (Lee and McPherron, 2001). Amthor et al (2004) also have proven that follistatin and Mstn communicate directly with high affinity during chick development, and Mstn inhibits terminal differentiation of muscle cells in high-density cell culture of limb mesenchyme and that follistatin rescues muscle differentiation in a concentration dependent manner. The results suggest that follistatin antagonizes Mstn by direct interaction, resulting in prevention of Mstn from executing its inhibitory effect on muscle development.

1.2.2. Physiological functions of myostatin in skeletal muscle growth

In addition to the inhibitory role of Mstn in myoblast proliferation and differentiation during development, numerous studies indicate that Mstn has a role in skeletal muscle growth and maintenance during the postnatal growth. Whittemore et al., (2003) produced monoclonal anti-Mstn antibody, and when they administered the antibody to mice, they observed that the antibody-treated mice gained approximately 10 % more weight than the control. In other study, the administration of monoclonal anti-Mstn antibodies to mdx mice, an animal model for Duchenne muscular dystrophy, increased body weight, whole muscle cross sectional area and muscle fiber area after 3 months of treatment as compared to control animals (Bogdanovich et al., 2002). Mstn suppression by anti-Mstn antibody generated via DNA vaccine method in mice increased skeletal muscle mass up to 31 %, as well as muscle function

(Tang et al., 2007). Conversely, systemic administration of Mstn by injection of Mstn-producing CHO cells into the thigh muscle or ectopic expression of Mstn through the electro transfer of Mstn-containing plasmid significantly decreased skeletal muscle mass in adult mice (Zimmers et al., 2002). Studies in which genetic manipulation was used to modify postnatal Mstn concentration also provided evidence supporting the role of Mstn in postnatal skeletal muscles. (Grobet et al., 2003) generated conditional Mstn knockout mice that demonstrated postnatal inactivation of Mstn. They reported that the conditional Mstn knockout mice had significantly increased muscle mass, as was demonstrated in the constitutive Mstn knockout mice generated by (McPherron et al., 1997). However, unlike the constitutive Mstn knockout mice, they reported that increase in skeletal muscle mass in the conditional Mstn knockout mice was primarily due to the hypertrophy of skeletal muscle fibers. Injection of plasmid expressing short interfering RNA (siRNA) targeting Mstn in adult mice increased tibialis muscle mass (10%) and fiber size (24%), along with a two-fold increase in satellite cell number (Magee et al., 2006). Direct injection of antisense RNA against Mstn also improved the muscle growth in adult normal and cachexia mice (Liu et al., 2008). Systemic overexpression of dominant negative form of Mstn in liver via adeno-associated virus method led to an increase in skeletal muscle mass in normal and dystrophic mice (Morine et al., 2010). Overexpression of dominant-negative latency-associated Mstn propeptide under the control of myosin light chain 1F promoter and 1/3 enhancer from the TSPY locus on the Y chromosome in transgenic mice induced a 5-20% increase in skeletal muscle mass (Pirottin et al., 2005). In support of the negative regulation of Mstn on postnatal muscle hypertrophy various studies reported a negative relationship between the level of Mstn and muscle mass under various physiological or pathological conditions that induced muscle loss or gain in many vertebrate species. (Carlson et al., 1999) examined Mstn mRNA abundance in mice gastrocnemius and plantaris muscles at 1 day after hind limb unloading but not at 3 or 7 days of hind limb unloading, suggesting that Mstn upregulation was associated with the atrophy of skeletal muscles. (Wehling et al., 2000) also reported that 10 days of unloading caused a 16% decrease in plantaris mass and a 110% increase in Mstn mRNA. Consistent with the above results, the expression of Mstn mRNA level increased 30-fold in both chronic and acute disuse-induced muscle atrophy (Reardon et

al., 2001). Mstn mRNA concentrations were significantly higher in skeletal muscles from the spaceflight rat than those in the ground-based controls, resulting in negative relationship between the Mstn mRNA expression and muscle mass (Lalani et al., 2000).

1.2.3. Myostatin signaling pathway

Like many other TGF- β superfamily growth factors, Mstn signals via two types of transmembrane serin/threonine kinase receptors; type 2 (*ActRIIB*) and type 1 (ALK-4 and / or ALK-5) located on the muscle cells (Lee and McPherron, 2001; Rebbapragada et al., 2003). Figure 1.1 illustrates current understanding of the Mstn signaling. Lee and McPherron (2001) generated transgenic mice overexpressing a dominant-negative form of *ActRIIB* that lacked kinase domain to examine the role for *ActRIIB* in mice. The transgenic mice demonstrated an increased muscle mass with individual muscles weighing up to 125% compared to those of wild-type mice, showing the involvement of *ActRIIB* in Mstn signaling. Furthermore, administration of soluble forms of *ActRIIB* receptors in mice appeared to have captured and prevented endogenous Mstn from binding to the *ActRIIB* receptors, leading to greater skeletal muscle mass that even exceeded the muscle mass of Mstn knockout mice (Lee et al., 2005). This finding not only confirms the role of *ActRIIB* in mediating Mstn signaling, but also suggests the existence of some unknown ligands, apart from Mstn, which may mediate through *ActRIIB* to inhibit muscle growth (Lee et al., 2005).

Most members of the TGF-B superfamily are believed to signal through Smad complex activated by heteromeric complexes of type 1 and type 2 serine/threonine kinase receptors (Mehra and Wrana, 2002). Most ligands binding to type 2 receptor activates type 1 receptor, resulting in the initiation of signal transduction pathways by phosphorylation of intracellular mediators such as Smad proteins. Eight different Smad proteins are divided into three functional groups; Smad 1, 2, 3, 5 and 8 belongs to the receptor regulated R-Smads, and Smad 4 belongs to the common partner Co-smad, and Smad 6 and 7 belongs to the inhibitory 1 Smads. R- Smads are phosphorylated by type 1 receptor serine kinase in response to the binding of TGF-B superfamily to tyep2 receptors. The Co-Smad positively regulates the phosphorylated R-Smads by hetero-oligomer formation with the R-Smads. The entire complex of R-

Smads and CO-Smad translocates into the nucleus. Inside nucleus, they interact with different cellular partners, bind to DNA, and regulate transcription of various downstream response genes. In contrast to R-Smads and Co-Smads, I-Smads inhibits the signaling of TGF-B superfamily proteins. I-Smads interact with type 1 receptors, thus compete with R-Smads for activation by the type 1 receptors. In addition, I-Smads also recruit Smurf ubiquitin ligases to catalyze degradation of the receptor complex. The transcription of I-Smads is known to be initiated by TGF-B superfamily proteins, thus providing a negative feedback regulation.

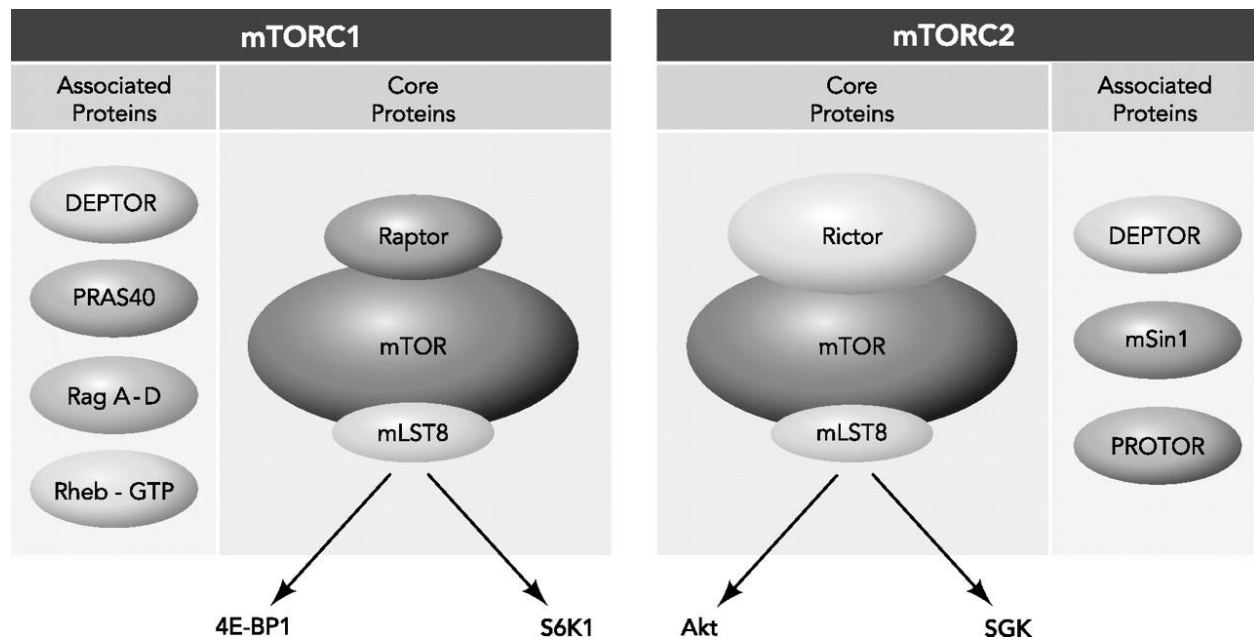
In support of the activation of Smad pathway by Mstn, (Langley et al., 2002) demonstrated that the addition of Mstn in muscle cell culture up-regulated the level of phosphorylated Smad3 during the myoblast differentiation, and the addition of anti-Mstn antibodies down-regulated the level of phosphorylated Smad3 in C2C12 cells (Bogdanovich et al., 2002). Moreover, Zhu et al., 2004 investigated the Mstn signaling pathway using a Mstn responsive p(CAGA)₁₂-MLP luciferase reporter assay system in which plasmids including various Smads were co-transfected. Their results showed that the myostatin-induced transcription required the participation of R-Smads (Smad 2/3) and Co-Smad (Smad4), and inhibitory Smad7 dramatically reduced the Mstn-induced transcription. These studies together demonstrate that Mstn binds to ActRIIB and activate type I activin receptor (ALK4 or 5), followed by Smad phosphorylation and nuclear translocation to mediate skeletal muscle mass regulation.

1.2.4. Interaction between the mTOR pathway and myostatin

Recently, studies show that there is a connection between the Smads signaling and the Akt/mammalian target or rapamycin (mTOR) pathway (Fig. 1.2). Sartori et al (2009) found an atrophy in muscle fibers that were transfected with constitutively active (c.a.) ALK4/5, a type 1 Mstn receptor, in mice, conforming the muscle atrophic effect of Mstn stimulation. To find out the involvement of Smad phosphorylation in the atrophic effect of ALK4/5 activation, they inhibited the Smad 2 and 4 activities through siRNA method in the atrophic effect of ALK4/5 activation. Their results showed that inhibition of Smad 2 and 3 could completely block ALK 4/5-mediated atrophy. Furthermore, induction of Akt could completely block the atrophic action of c.a. ALK 4/5, indicating that Mstn signaling involves the Akt

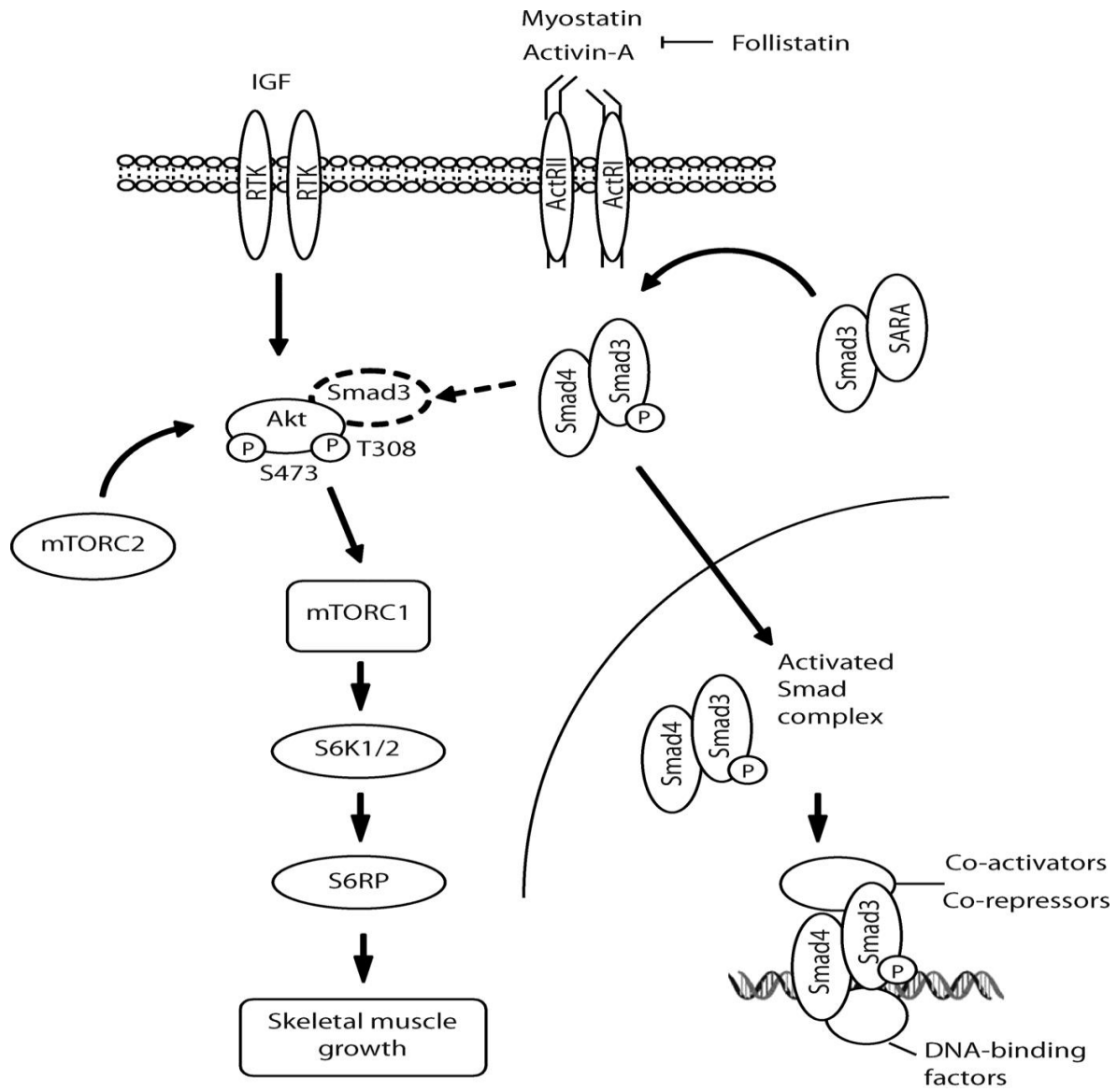
downstream of Smad regulation. It was also reported that Akt phosphorylation was inhibited by Mstn in human myotube, and the effect was dependent upon the presence of Smad2 and 3 (Trendelenburg et al., 2009). Blockade of the Akt/mTOR pathway, using small interfering RNA to regulatory-associated protein of mTOR (RAPTOR), increased Mstn-induced Smad phosphorylation, establishing a feed-forward mechanism. Mstn activates Smad2, leading to inhibition of TORC1 via Akt inhibition, which in turn potentiates Mstn's activation of Smad2 (Trendelenburg et al., 2009). Addition of IGF-1 dominantly blocked the effects of Mstn when applied to either myoblasts or myotubes (Trendelenburg et al., 2009). Moreover, significant increase in Akt mRNA expression, protein level, phosphorylation and activity was observed along with increase in S6 and S6K protein level in Mstn knockout mice compared with wild type mice (Morissette et al., 2009; Lipina et al., 2010; Hitachi et al., 2014). These results together indicate that Akt is a particular crossing point between the IGF-1 and Mstn signaling and that IGF-1 can rescue the activation of the PI3k/Akt pathway that is blunted by Mstn. Recently, it has been reported that the expression of micro RNA, miR486, was significantly increased in Mstn knockout mice compared with wild type mice (Hitachi et al., 2014). Furthermore, their study showed that the miR486 regulated the level of Akt probably via the control of PTEN, a phosphatase involved in the suppression of Akt signaling, thus suggesting that miR486 is potentially an intermediary molecule connecting Mstn signaling and the Akt/mTOR pathway.

Since RAP suppresses the activity of mTORC1, it is assumed that if the Akt/mTOR pathway is the major signaling pathway employed by Mstn for its regulation of skeletal muscle mass, then RAP administration to Mstn-pro mice, which are characterized by hypermuscular phenotype vis genetic suppression of Mstn, will block the muscle hypertrophy of Mstn-pro mice. Therefore, this study was designed to investigate the effect of RAP administration on muscle growth of Mstn-pro mice and to examine the effect of RAP on the regulation of signaling molecules in mTOR pathway in skeletal muscles of Mstn-pro mice.



From Frost and Lane 2011

Figure 1.1. Diagram illustrating the mTOR complex 1 and mTOR complex 2.



From Winbanks et al., 2012

Figure 1.2. Diagram illustrating the Mstn and Akt/mTOR signaling pathway.

CHAPTER 2

MUSCLE HYPERTROPHY INDUCED BY MYOSTATIN INHIBITION IS SUPPRESSED BY RAPAMYCIN ADMINISTRATION

2.1. ABSTRACT

Recent studies have indicated that myostatin (Mstn), a skeletal muscle specific negative growth factor, regulates skeletal muscle mass through the anabolic mechanical target of rapamycin (mTOR) pathway. The mTOR pathway is known to be blocked by rapamycin (RAP), thus a study was designed to examine the effect of RAP administration on muscle growth in Mstn-propeptide transgenic mice, a hypermuscular phenotype by Mstn inhibition. 5 week old male heterozygous Mstn-propeptide transgenic mice and wild type littermates were administered with 0 or 3 mg/kg body weight of RAP intraperitoneally every other day for 4 weeks. At the end of RAP treatment, animals were sacrificed, and gastrocnemius, plantaris, and soleus muscles were dissected, weighed, and snap-frozen for later analysis. Body weight gain of transgenic mice was greater than that of wild type mice ($P < 0.01$). RAP suppressed body weight gain about 40% in both genotypes ($P < 0.05$). RAP also suppressed muscle growth in both genotypes, but the extent of suppression was greater in transgenic mice than in wild type mice ($P < 0.05$). Real time PCR analysis showed that myogenic regulatory factors including MyoD and myogenin were not affected either by genotype or RAP. Myf5 was down-regulated by RAP, but not affected by genotype. Also, Mrf4 was down-regulated by both RAP and genotype. Signaling molecules of the mTOR pathway, including Akt, p70S6 kinase and 4E-BP1, were up-regulated in transgenic mice. Rap treatment did not affect the expression of the mTOR signaling molecules in wild type mice, but significantly down-regulated the expression of those genes in transgenic mice. Results of this study show that the mTOR pathway is involved in the regulation of muscle growth by Mstn.

2.2. INTRODUCTION

Myostatin (Mstn), also known as growth and differentiation factor-8 (GDF-8), is a member of the transforming growth factor-beta (TGF- β) superfamily and negatively regulates skeletal muscle mass (McPherron et al., 1997; Lee and McPherron, 2001). For example, several studies have shown that

dysfunctional mutation of Mstn gene brought dramatic muscle mass improvement in cattle (Kambadur et al., 1997; Grobet et al., 1998), dogs (Mosher et al., 2007), sheep (Clouet et al., 2006) and humans (Schuelke et al., 2004). Mstn regulates muscle growth in two different ways: one way is by controlling muscle fiber number during development, and the other way is by controlling hypertrophy of postnatal skeletal muscle fibers.

Mstn binds to ActRIIB receptor for its signaling, leading to muscle growth regulation (Lee and McPherron, 2001; Rebbapragada et al., 2003). Transgenic mice overexpressing a dominant-negative form of ActRIIB that lacked kinase domain demonstrated increased muscle mass with individual muscles weighing up to 125% of those of wild-type mice, supporting the involvement of ActRIIB in Mstn signaling (Lee and McPherron, 2001). Like other members of the TGF- β superfamily, Mstn appears to signal through Smad complex activated by heteromeric complexes of type 1 and type 2 serine/threonine kinase receptors and trans-nuclear location of the activated Smad complex, followed by transcription of target genes. In support of the activation of Smad pathway by Mstn, it has been demonstrated that the addition of Mstn in muscle cell culture up-regulated the level of phosphorylated Smad3 during the myoblast differentiation (Langley et al., 2002), and the addition of anti-Mstn antibodies down-regulated the level of phosphorylated Smad3 in C2C12 cells (Bogdanovich et al., 2002). Moreover, (Zhu et al., 2004) showed that the Mstn-induced transcription required the participation of R-Smads (Smad 2/3) and Co-Smad (Smad4). The Smad signaling pathway activated by Mstn has been well established, but there is little information regarding the molecular mechanism(s) by which Smads activation is being connected to muscle protein metabolism.

Mammalian target of rapamycin (mTOR) pathway is regarded as a key regulator of cell growth. The mTOR pathway integrates signals from growth factors, nutrients, and energy status to control protein synthesis and other cell functions (Asnaghi et al., 2004). Recent studies show that the mTOR pathway plays an important role in muscle growth (Bodine et al., 2001; Pallafacchina et al., 2002). The role of the mTOR pathway in muscle growth was demonstrated by *in vivo* studies, in which the hypertrophic responses induced by overload or regenerating muscle growth are blocked by rapamycin, an mTOR

suppressing molecule (Pallafacchina et al., 2002). Recent studies suggest that there is a connection between the Smads signaling and the Akt/mechanistic target of rapamycin (mTOR) pathway. Muscle fiber atrophy was observed in mice transfected with constitutively active (c.a.) ALK4/5, a type I Mstn receptor (Sartori et al., 2009). To examine the involvement of Smad phosphorylation in the atrophic effect of ALK4/5 activation, the above authors suppressed the Smad2 and 3 activities through siRNA method in the c.a. ALK transfected muscle fiber. Their results showed that inhibition of Smad 2 and 3 could completely block ALK4/5-mediated atrophy. Furthermore, induction of Akt could completely block the atrophic action of c.a. ALK4/5, indicating that Mstn signaling involves the Akt suppression by Smad activation. It was also reported that Akt phosphorylation was inhibited by Mstn in human myotubes, and the effect was dependent upon the presence of Smad2 and 3 (Trendelenburg et al., 2009).

In the current study, it was hypothesized that if the regulation of muscle mass by Mstn is via the inhibition of Akt/mTOR pathway, the muscle hypertrophy of transgenic mice overexpressing Mstn propeptide will be suppressed by rapamycin, an mTOR suppressor. Therefore, the objective of this study was 1) to examine the effect of rapamycin administration on muscle growth of transgenic mice engineered to induce hypermuscularity via Mstn propeptide overexpression, and 2) to examine the effect of rapamycin on the regulation of signaling molecules in mTOR pathway.

2.3. MATERIALS AND METHODS

2.3.1. Animals and sample collection

All the procedures of animal care were approved by the Institutional Animal Care and Use Committee at the University of Hawaii (protocol #, 13-1597). The mice were housed in the Small Animal Facility, UH Manoa with a 12 h light /dark cycle and maintained in a temperature and humidity controlled condition. Mice had free access to feed (Lab Diet # 5001 Rodent Diet, Purina Mills, Richmond, IN) (10 % kcal fat, ME 3.85 kcal/g) and clean water. We used a transgenic mouse strain (Mstn-pro) which is characterized by a postnatal hyper-muscularity due to suppression of Mstn activity by overexpressing Mstn propeptide (Yang et al., 2001). Wild type male B6SJL F1 mice and Mstn-pro transgenic female mice were mated to produce heterozygote Mstn-pro transgenic and wild-type litter mate genotypes. Pups

were weaned at 4 weeks of age, and tail tissues from mail pups were collected at the time of weaning for genotyping. After genotyping, male mice were separated by their genotypes, and each genotype divided into two groups (0 or 3 mg/kg body wt of rapamycin). Rapamycin administration started at 5 weeks of age. 400 µg of rapamycin was dissolved in 10 µl of DMSO, and the rapamycin stock in DMSO was suspended with 990 µl of PBS. Rapamycin was intraperitoneally administered every other day for 4 weeks, and body weight and grip strength were measured two times a week. After the administration, animals were sacrificed by CO₂ asphyxiation, and gastrocnemius, plantaris, and soleus muscles of the hind legs were collected, weighed, and frozen in liquid nitrogen and stored at -80 °C until analysis.

2.3.2. Genotyping

DNA was extracted from the tail tissue by phenol/chloroform extraction after overnight digestion at 50°C in a Tris buffer (1 M, pH 8.0) containing 10 % sodium dodecyl sulfate (SDS), 0.5 M ethylenediaminetetraacetic acid (EDTA), and proteinase K (0.7 mg/ml). The extracted DNA was amplified with a primer set specific for transgenic mice by polymerase chain reaction (PCR). The forward sequence of primer was 5' – GACAGCAGTGATGGCTCT-3' and the reverse was 5' - CTTGTCATCGTCGTCCTTGTAAATCGGTAC - 3', and the PCR condition was the same as previously reported (Yang et al., 2001). The PCR product was subjected to 1 % agarose gel electrophoresis with ethidium bromide staining to examine the presence of a transgenic PCR product.

2.3.3. Real-time quantitative PCR (qPCR)

Total RNA was isolated using the Trizol reagent (Invitrogen, CA, USA) following the manufacture's protocol. Briefly, 1 ml of Trizol was added to 2 mg of hind limb muscle samples and homogenized and left for 5 minutes at room temperature. 0.2 ml of chloroform per 1 ml of Trizol was added and vortexed for 15 seconds. After incubation for 3 minutes at room temperature, the samples were centrifuged at 12,000 x g for 15 minutes at 4 °C. Upper layer of the aqueous phase was transferred into a new tube, and 0.5 ml of 100% isopropanol was added. After the incubation for 10 minutes at room temperature, the samples were centrifuged at 12,000 x g for 10 minutes at 4 °C. Supernatant was removed from the tube, and the RNA pellet was washed with 1 ml of 75% ethanol. After vortexing briefly, the tube

was centrifuged at 7500 x g for 5 minutes at 4 °C. Ethanol was discarded and the pellet was air-dried for 5 - 10 minutes. The RNA pellet was suspended with 50 µl of RNase-free water. Total RNA was treated with 1 µl of RNase free- DNase I (Thermo Scientific, Hudson, NH, USA) to prevent contamination from any DNA. Total RNA was used to generate the cDNA by using Transcriptor Reverse Transcriptase (Roche Applied Science, Mannheim, Germany). 10 µM of Oligo (dT)15 primer was added to 1 µg of RNA with final volume being adjusted to 20 µl by adding DNase-, RNase-free water, and incubated at 65°C for 10 minutes. After incubation, 4 µl of 5 x Transcriptor RT Reaction Buffer, 2 µl of 10 mM dNTP, and 0.5 µl of Transcriptor Reverse Transcriptase were mixed well and incubated for 30 minutes at 55°C. The activation of Transcriptor Reverse Transcriptase was stopped by incubating at 85 °C for 5 minutes. Synthesized cDNA was ten times diluted with nuclease free water and ready for real time PCR (qPCR).

The expression level of genes, including MyoD, myf5, myogenin, MRF4, Akt, 4EBP-1, p70S6k, and Mstn were estimated by the real time quantitative PCR using the above cDNA. The sequences of primer sets of each gene was designed to yield ~150 bp and is shown in Table 2.1. The agarose gel analysis of PCR products of each gene is shown in Fig. 2.4. Real time PCR reaction was prepared with SYBR® Select Master Mix (Applied Biosystems, Foster City, CA). Each reaction included 2 µl of diluted cDNA, 10 µl of 2 x concentration SYBR Green I Master, 0.5 µl of 10 pM primer, and Nuclease free water. The real time PCR (qPCR) was run on Applied Biosystems 7300 Fast Real-Time PCR (Applied Biosystems, Foster City, CA) following the manufacturer's instruction. The cycling conditions were 1 cycle of denaturation at 94 °C for 4 min and amplification step was performed as above for 42 cycles: 94°C for 40 s, 60°C for 30 s, and 72°C for 30 s. GAPDH was used for endogenous gene. The comparative CT method was used for data analysis, The level of target gene was normalized to endogenous control gene (GAPDH gene) by subtracting the CT values of respective GAPDH gene from the CT values of target gene. The CT values were analyzed by two-way ANOVA using Prism 6 software (Graphad, San Diego, CA).

2.3.4. Western blot analysis of Akt, 4E-BP1 and p70s6k

For Western blot analysis, we used the procedure described previously by Dreyer et al. (2006) . Muscle tissue samples were transferred to 1 ml of ice-cold lysis buffer [50 mM Tris –HCl (ph 7.4), 250 mM mannitol, 50 mM NaF, 5 mM Na pyrophosphate, 1mM EDTA, 1 mM ethylene-glycol tetraacetic acid (EGTA), 1 % Triton X-100, 1 mM dithiothreitol (DTT), 1 mM benzamidine, 0.1 mM phenylmethylsulfonylfluoride (PMSF), and 5 µg/ml soybean trypsin inhibitor] and homogenized at 4 °C. The homogenized samples were centrifuged at 6,000 g for 10 min at 4°C, and aqueous layer of the supernatants were saved for Western blot analysis of Akt and p70S6k. For the 4E-BP1 Western blot analysis, 50 µl of the above the supernatants were incubated at 100 °C for 10 min, centrifuged for 30 min at 10,000 g at 4 °C, and the supernatants were saved. The concentration of protein in supernatants was analyzed using the modified Lowry protein assay kit (Pierce, Rockford, IL) to estimate protein content. 11.2 µg of extracted proteins mixed with Bromophenol Blue (BioRad, Hercules, CA) were loaded to 12.5 % SDS-PAGE gel and then proteins were transferred to polyvinilidene difluoride (PVDF) membranes. The membranes were blocked for 2 hours at room temperature in Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl at pH 7.5) containing 5% nonfat dry milk and then incubated overnight with primary antibody in Tris-buffered saline plus 0.1% Tween - 20 (TBST, 20 mM Tris – HCl, 150 mM NaCl at pH 7.5) at 4°C. The primary antibodies were rabbit anti-Akt (1:1,000; Cell signaling, Beverly, MA), rabbit anti-p70S6k (1:1,000; Cell signaling), rabbit anti-4E-BP1 (1:1,000; Cell signaling), rabbit anti-phopho-p70S6k (Thr389; 1:1,000; Cell signaling), and rabbit anti-phopho-4E-BP1 (Thr 37/46; 1:1,000; Cell signaling) were used to detect protein expression levels. The membranes were washed three times with TBST and incubated with horseradish peroxidase-conjugated anti-rabbit antibody (1:25000, Invitrogen, Carlsbad, CA) in TBST for 1 hr 30 minutes at room temperature. After washing with TBST, the membrane was developed with the enhanced chemiluminescent reagent (ECL plus Western Detection; GE Healthcare Biosciences; Pittsburg,PA), followed by apposition of the membrane to auto-radiographic films (Hyperfilm ECL; Kodak, CA).

2.3.5. Statistical analysis

All values were expressed as mean \pm SEM and P values less than 0.05 were chosen as statistically significant. Two-way analysis of variance (ANOVA) was used to analyze the effect of genotype, RAP, and interaction on body and muscle weights, as well as the levels of gene expression.

2.4 RESULTS

2.4.1. Effects of RAP on body weights of wild type and Mstn-pro mice

The effect of RAP administration on body weights is summarized in Fig 2.1. Mstn-pro mice were heavier ($p < 0.05$) than wild type mice before the RAP administration (19.5 vs 18.6 g). Body weight gain of the wild type mice during the 4-week period was 23% and 17% in PBS control and RAP administration, respectively, and the body weight gain of the Mstn-pro transgenic mice with or without RAP was 33% and 18%, respectively. The results demonstrate that Mstn-pro transgenic mice grew significantly faster ($p < 0.001$) than wild type mice during 4 weeks of the experimental period without RAP administration. This result confirmed that RAP administration suppressed the growth of both wild type and Mstn-pro transgenic mice. In the wild type mice, the growth suppression by RAP was 6% while growth suppression by RAP administration was 15% in Mstn-pro mice. Therefore, this results demonstrate that the extent of growth suppression by RAP administration is greater in Mstn-pro than that in wild type mice. However, the interaction was not significant on body weight (Not shown in the figure).

2.4.2. Effects of RAP administration on muscle and organ weights of wild type and Mstn-pro mice

Table 2.2. Summarizes the effect of RAP on muscle weights of wild type and Mstn-pro mice. Soleus, plantaris, and gastrocnemius muscles of Mstn-pro mice were heavier ($p < 0.01$) than those of the wild type. Percentage of those muscle weights to body weights of Mstn-pro was also higher than those of the wild type. RAP suppressed muscle weights of plantaris ($p < 0.1$) and gastrocnemius muscle ($P < 0.01$) in both groups, but the percentage of these muscles was not affected by RAP administration. The suppression of plantaris and gastrocnemius muscle weight by RAP administration in wild type mice were 4.6% and 13.8%, respectively, while the suppression of those muscle weight in Mstn-pro mice were 13.3% and 17.9%, respectively. Thus, it appears that the extent of muscle weight suppression by RAP was larger

in *Mstn*-pro mice than in wild type mice. However, there was no interaction effect on muscle weight.

Table 2.3. Shows the effect of RAP administration on organs weights of wild type and *Mstn*-pro mice. Genotype did not affect the weights of heart, liver, spleen, kidney, white fat, and brown fat. RAP administration decreased the weights of spleen and kidney in both genotypes, but has no influence on the weights of heart, liver, white fat, and brown fat. Also, there was no interaction effect on organs (Not shown in the figure).

2.4.3. Effects of RAP on grip strength of wild type and *Mstn*-pro mice

The effect of RAP administration on grip strength of wild type and *Mstn*-pro mice is shown in Fig. 2.2. The mean grip strengths of *Mstn*-pro mice were generally greater than those of wild type mice during the experimental period, but statistically significance was only observed at the last two measurement time points (24 and 28 days). RAP treatment also appeared to suppress the mean grip strength of both genotypes throughout the experimental period, but statistically significance was only shown at 28 days. There was no interaction effect on grip strength (Not shown in the figure).

When we examined the grip strength per unit body weight (grip strength/body weight), the grip strength/body weight was not affected by either genotype or RAP administration (Fig. 2.3) Also, there was no effect on interaction (Not shown in the figure).

2.4.4. Expression levels of myogenic factors and Akt/mTOR pathway

We analyzed the expression of myogenic regulatory factor genes and genes involved in the Akt/mTOR signaling pathway to examine whether the expression of these genes are by the transgene and RAP administration. Amplification plots and dissociation curves are shown Fig. 2.5, and the result indicated that the real time PCR conditions and primers worked well for the analysis (Fig 2.4).

Fig. 2.6 is showing the expression of myogenic regulatory factor genes as affected by RAP in wild type and *Mstn*-pro mice. No genotype and RAP effects were found on both MyoD and myogenin gene expression. RAP suppressed myf 5 gene expressions in both genotype of mice, but genotype did not affect myf5 gene expression (Fig. 2.6C). Mrf4 gene has higher level of gene expression in wild type mice compared to *Mstn*-pro mice, and RAP suppressed its expression in both genotypes (Fig. 2.6D).

Fig. 2.7. shows how the expression of mTOR pathway-related genes, including Akt, p70S6K, and 4E-BP1, were regulated by genotype and RAP administration. The expressions of Akt, p70S6K, and 4E-BP1 in Mstn-pro mice were significantly higher than those of wild type mice. RAP administration did not affect the expressions of those genes in wild type mice, but significantly suppressed the expression of those genes in Mstn-pro mice.

Myostatin gene expression tended to be lower ($P < 0.1$) in wild type mice as compared to Mstn-pro mice (Fig 2.8). However, there is no effect of RAP administration in Mstn gene expression in both wild type and transgenic mice.

2.4.5. Validation of western blotting

Prior to Western blot analysis of the level of phosphorylation of Akt and p70S6k, the binding of primary antibodies to their target proteins was examined. All the primary antibodies demonstrated their binding to their target proteins, including P70S6k (70 kDa), P-P70S6k (70 kDa), Akt (60 kDa), p-Akt (60 kDa), and GAPDH (37 kDa), prepared from fresh frozen muscle samples (Fig. 2.9). After the validation of the Western blot procedure, frozen muscle samples were homogenized in the buffer solution described in the Materials and Methods section, followed by centrifugation for collection of supernatant and storage at -20 for later use in Western blot analysis. When the frozen supernatant was thawed for Western blot analysis, supernatant was not completely solubilized, thus the thawed sample solution was subjected to centrifugation to use the supernatant for Western blot analysis. Western blot with the supernatant solution prepared this way did not show any binding (data not shown). Since it was suspected that sample preparation, particularly the freezing of supernatant of homogenized sample, affected the negative outcome, samples from fresh tissue and previously-prepared and frozen were subjected to Western blot analysis (Fig. 2.10).

Newly prepared samples (lanes 1 and 2) were shown the presence of Akt, P-Akt, p70S6k, and GAPDH, while the previously prepared and freezer stored samples only showed the presence of GAPDH. To examine whether Akt and p70S6k are in the pellet fraction of the previously-prepared and freezer stored samples, Western blot was performed with whole thawed sample that were rehomogenized (Fig

2.11). No presence of Akt and p-Akt was observed in both samples., suggesting that the sample preparation scheme rendered the Akt and p-Akt losing their affinity to anti-Akt and anti-p-Akt antibodies. Therefore, Western blot data could not be collected.

2.5. DISCUSSION

The anabolic mechanistic target of rapamycin (mTOR) pathway, which is blocked by rapamycin (RAP), is regarded as a key regulator of cell growth, and recent studies show that the mTOR pathway plays an important role in skeletal muscle growth (Bodine *et al.*, 2001; Pallafacchina *et al.*, 2002). Some studies have indicated that Mstn regulates skeletal muscle mass through the mTOR pathway. For example, Mstn administration suppressed Akt phosphorylation in human primary myotube and mouse C2C12 derived myotubes (Trendelenburg *et al.*, 2009). Transient genetic overexpression of Mstn decrease the phosphorylation of various signaling molecules responsible for the Akt/mTOR pathway, including Akt, TSC2, S6, and 4E-BP1 (Amirouche *et al.*, 2009). Mstn overexpression by electro transfer of Mstn-expression vector in mice skeletal muscle also decreased in Akt, 4E-BP1 phosphorylation (Wang and Proud, 2006). These studies together suggest that Mstn regulate the Akt/mTOR signaling pathway by post-translational modulation (phosphorylation) of involved signaling molecules. However, not all studies agree with the Mstn modulation of Akt/mTOR pathway for skeletal muscle mass regulation. For example, Mstn suppression with the administration of anti-Mstn antibody in mice did not affect the phosphorylation of Akt and 4E-BP1 (Welle *et al.*, 2009). Since RAP suppresses the mTOR pathway, in this study, we administered RAP to Mstn-pro mice, which exhibit hypermuscular phenotype via overexpression of Mstn blocker (Mstn propeptide), to examine how the expression of mTOR signaling molecules, as well as muscle mass, are affected.

The current results show that expression of Akt, 4E-BP1 and p70S6k in Mstn-pro mice were all significantly higher (3-8 fold) compared with wild type mice, indicating that Mstn can modulate the Akt/mTOR pathway at the transcriptional level. Similarly, Morissette *et al.* (2009) reported that genetic loss of Mstn increased Akt gene expression, protein levels of Akt and phosphorylated Akt, and Akt activity. The same study also reported that the level of phosphorylated S6 was increased by the genetic

loss of Mstn even though the protein level of S6 was not affected. In the same animal model, Lipina *et al.* (2010) also observed that genetic loss of Mstn an increased protein levels of Akt and phosphorylated Akt. However, unlike the result of Morissette *et al.* (2009), they observed an increase in proteins levels of both S6, and S6K in skeletal muscle of Mstn-knockout mice. Conversely, overexpression of Mstn in skeletal muscle did not affect the protein levels of Akt, TSC2, and mTOR, while phosphorylation of Akt, TSC2, mTOR, and S6, and 4E-BP1 was significantly decreased by the Mstn overexpression (Amirouche *et al.* 2009). Using a muscle cell culture system, it has been observed that Mstn administration increased the phosphorylation of Akt without a change in the level of Akt (McFarlane *et al.* 2006; Trendelenburg *et al.* 2009). Many of these results, thus, suggest that Mstn regulates the Akt/mTOR pathway primarily via post-translational modification of involved signaling molecules rather than transcriptional modification. However, our current results together with those of Morrissette *et al.* (2009) and Lipina *et al.* (2010) indicate that Mstn can also exert transcriptional regulation of the Akt/mTOR pathway.

The current results shows that the administration of RAP had no significant effect on skeletal muscle mRNA expression of Akt, p70S6k and 4E-BP1 in wild type mice, while RAP administration significantly suppressed the expression of these genes in Mstn-pro mice. The mechanism for activating the Akt/mTOR pathway mostly involves the phosphorylation of signaling molecules in the pathway (Laplante & Sabatini 2012), and many studies reported that skeletal muscle hypertrophy induced by resistant exercise was associated with increased phosphorylations of Akt/mTOR signaling molecules without much effect on the total levels of these proteins (Baar & Esser 1999; Dreyer *et al.* 2008; Glover *et al.* 2008; O'Neil *et al.* 2009). It is, thus, speculated that the decreased in skeletal muscle mass by RAP administration in wild type mice were probably through the suppression of the phosphorylation of signaling molecules in the Akt/mTOR pathway, but not by suppression of the transcription of these molecules. In support of the speculation, it has been reported that chronic administration of RAP in rat suppressed the phosphorylation of Akt as well as S6 independent of total protein level of the molecules (Deblon *et al.* 2012). In other study, chronic RAP administration up to 6 weeks suppressed Akt, mTOR S6K and 4E-BP1 phosphorylation in liver without changes in the levels of these proteins (Fang *et al.*

2013). In contrast to wild type mice, in *Mstn*-pro mice, the RAP administration suppressed the expression of Akt, p70S6k and 4E-BP1. Presumably, the suppression of the expression of Akt, p70S6k and 4E-BP1 induced a much far greater suppression of the Akt/mTOR pathway by RAP administration in *Mstn*-pro mice than in wild type mice. Then it is quite likely that the much greater suppression of muscle mass by RAP administration in *Mstn*-pro mice, thus, is related to the greater suppression of the Akt/mTOR pathway. As far as we know, this is the first observation showing that in conditions of genetic *Mstn* suppression, RAP administration can work at the transcriptional level to suppress the Akt/mTOR signaling pathway, thus future studies need to look at that mechanism(s), cellular or physiological context are involved in the RAP induced transcriptional regulation of the Akt/mTOR pathway.

Mstn's regulation of skeletal muscle growth involves skeletal muscle hyperplasia primarily during development and muscle hypertrophy, which occurs during postnatal muscle growth (Lee 2004). In vitro muscle cell culture system, *Mstn* has shown to suppress cell proliferation and differentiation through the activation of cell cycle inhibitors (Thomas *et al.* 2000; Langley *et al.* 2002; Rios *et al.* 2002; Joulia *et al.* 2003), and it has been shown that the inhibition is associated with either decreasing level of myogenic regulatory factors, such as MyoD (Langley *et al.* 2002; McFarlane *et al.* 2006) and myogenin (Joulia *et al.*, 2003). In vivo, McFarlane *et al.* (2006) showed that overexpression of *Mstn* in mice also decrease the MyoD expression. Conversely, in doubled muscle cattle, which expresses non-functional mutant *Mstn*, it has been reported that fetal skeletal muscle expressions of MyoD and myogenin was significantly increased during the fetal development, suggesting the role of *Mstn* on the expression of myogenic regulatory factors. Even though myogenic regulatory factors play an important role in the growth and maintenance of post-natal skeletal muscle, very little is known about how *Mstn*-influence myogenic regulatory factors during its regulation of skeletal muscle mass at postnatal stage. Our current results show that there is no difference in the post-natal skeletal muscle expressions of MyoD, Myf5, and myogenin between wild type and *Mstn*-pro mice, but *Mrf4* expression was significantly decreased in *Mstn*-pro mice compared with wild type mice. Interestingly, RAP administration did not affect the expression of MyoD and myogenin in both genotype, but significantly decreased the expression of both

Myf5 and Mrf4. Myogenic regulatory factors, including MyoD, Myf5 and myogenin play a role in muscle determination, differentiation, while Mrf4 is mostly expressed after birth and play a role in the maintenance of skeletal muscles (Perry & Rudnick 2000). It has been shown that in postnatal skeletal muscles, Mstn-induced skeletal muscle growth occurs without satellite cell proliferation and differentiation (Lee *et al.* 2012), suggesting that elevated activation of MyoD, myogenin and Myf5 may not be required during postnatal muscle hypertrophy induced by Mstn. The absence of effect on transcription of MyoD, Myf5, and myogenin by genetic suppression of Mstn, thus, is in line with the above finding (Lee *et al.* 2012). In support of the role of Mrf4 in the postnatal maintenance of skeletal muscles, studies have shown that Mrf4 expression is downregulated during disuse atrophy or elevated during stretch-induced hypertrophy (Loughna & Brownson 1996; Lowe *et al.* 1998). In this context, the downregulation of Mrf4 by RAP administration was somewhat expected, but the suppression of Mrf4 in Mstn-pro mice was totally unexpected. It can be speculated that the enhanced production of skeletal muscle proteins induced by Mstn suppression potentially activated negative feedback mechanism. There is little information regarding the presence of negative feed back regulation on myogenic regulatory factors, and future studies may need to consider to examine the issue.

The expression level of Mstn gene was higher in Mstn suppressed mice compared to wild type mice. This may because of intracellular negative feed back loop regulated the myostatin gene expression in Mstn-pro mice.

In conclusion, the result of this study shows that RAP suppressed the muscle mass increase and skeletal muscle expression of genes in the Akt/mTOR pathway in transgenic animals whose Mstn activity was genetically suppressed. This result, thus, support that Mstn suppresses skeletal muscle mass by suppression of the Akt/mTOR pathway. This study only examined the level of gene expression of the Akt/mTOR signaling molecules. Since the level of gene expression is not always associated with increased protein level, future studies need to examine the level of protein and phosphorylation of these proteins in the Akt/mTOR pathway as affected by RAP treatment.

Table 2.1. Sequence of PCR primers of genes used for real-time PCR

Gene	RefSeq	Forward primers (5'-3')	Reverse Primers (5'-3')
Gapdh	NM_001289726	ACCCAGAAGACTGTGGATGG	CACATTGGGGGTAGGAACAC
MyoD	NM_010866	GACAGGGAGGAGGGGTAGAG	TGCTGTCTCAAAGGAGCAGA
Myogenin	NM_031189	CTGCCTAAAGTGGAGATCCTG	TGGGAGTTGCATTCACTGG
Mrf4	NM_008657	CCCTAGAGCTACAAACCCAAG	GCTGAGGCATCCACGTTTG
Myf5	NM_008656	AGGAAAAGAAGCCCTGAAGC	GCAAAAAGAACACGCAGAGG
Akt	NM_005163	GCCCTCAAGTACTCATTCCAG	ACACAATCTCCGCACCATAG
P70S6k1	NM_001114334	TGAGTCAAGCCTTGGTCGAG	AAGAGTCGAGAGAGACGCCC
4E-BP1	NM_007918	CGGAAGATAAGCGGGCAG	CAGTGTCTGCCTGGTATGAG
Myostatin	NM_010834	TGCAAAATTGGCTCAAACAG	GCAGTCAAGCCCAAAGTCTC

Table 2.2. Muscle and body weights in wild type and Mstn-pro mice as affected by RAP administration

	Wild type		Mstn-pro		Significance		
	0 mg	3 mg	0 mg	3 mg	GT	RAP	GT x RAP
Number of animals	11	10	7	7			
Initial body, g	18.5 (0.38)	18.6 (0.38)	19.9 (0.45)	19.1 (0.27)	*	NS	NS
Final body, g	23.3 (0.55)	22.2 (0.47)	26.1 (0.47)	22.8 (0.53)	***	***	NS
Soleus, mg	13.6 (0.91)	12.0 (0.61)	15.9 (0.62)	15.2 (0.89)	**	NS	NS
% Soleus	0.060 (0.0040)	0.056 (0.0031)	0.061 (0.0021)	0.070 (0.0053)	+	NS	NS
Plantaris, mg	26.3 (0.93)	25.1 (0.83)	44.0 (2.65)	38.0 (3.43)	***	+	NS
% Plantaris	0.12 (0.006)	0.12 (0.004)	0.17 (0.009)	0.17 (0.012)	***	NS	NS
Gastrocnemius, mg	228.5 (10.24)	197.0 (5.90)	331.8 (16.23)	272.3 (21.78)	***	**	NS
% Gastrocnemius	1.00 (0.043)	0.93 (0.021)	1.27 (0.047)	1.23 (0.067)	***	NS	NS

Values are means (\pm SEM). NS, not significant; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; +, $P < 0.1$; NS, not significance. GT, genotype; RAP, rapamycin.

Table 2.3. Organ weights in wild type and Mstn-pro mice as affected by RAP administration

	Wild type		Mstn-pro		Significance		
	0 mg/kg	3 mg/kg	0 mg/ kg	3 mg/kg	GT	RAP	GT x RAP
Heart Wt, g	0.126 (0.0053)	0.134 (0.0084)	0.139 (0.0047)	0.127 (0.0089)	NS	NS	NS
% Heart	0.55 (0.018)	0.62 (0.031)	0.53 (0.015)	0.57 (0.030)	NS	*	NS
Liver Wt, g	1.33 (0.031)	1.48 (0.049)	1.46 (0.103)	1.34 (0.083)	NS	NS	*
% Liver	5.84 (0.087)	6.94 (0.147)	5.56 (0.296)	6.05 (0.178)	**	***	NS
Spleen Wt, g	0.070 (0.0036)	0.060 (0.0077)	0.064 (0.005)	0.047 (0.005)	NS	*	NS
% Spleen	0.31 (0.013)	0.28 (0.032)	0.25 (0.023)	0.21 (0.016)	*	NS	NS
Kidney Wt, g	0.45 (0.012)	0.43 (0.015)	0.48 (0.020)	0.42 (0.024)	NS	*	NS
% Kidney	1.98 (0.056)	2.01 (0.076)	1.84 (0.043)	1.92 (0.070)	+	NS	NS
White fat Wt, g	0.19 (0.026)	0.27 (0.030)	0.21 (0.034)	0.23 (0.055)	NS	NS	NS
% White fat	0.96 (0.133)	1.26 (0.149)	0.80 (0.127)	1.03 (0.224)	NS	NS	NS
Brown fat Wt, g	0.10 (0.004)	0.11 (0.007)	0.11 (0.005)	0.10 (0.008)	NS	NS	NS
% Brown fat	0.42 (0.013)	0.53 (0.034)	0.43 (0.024)	0.46 (0.026)	NS	*	NS

Values are means (\pm SEM). NS, not significant; ***, P < 0.001; **, P < 0.01; *, P < 0.05; +, P < 0.1; NS, not significance. GT, genotype; RAP, rapamycin.

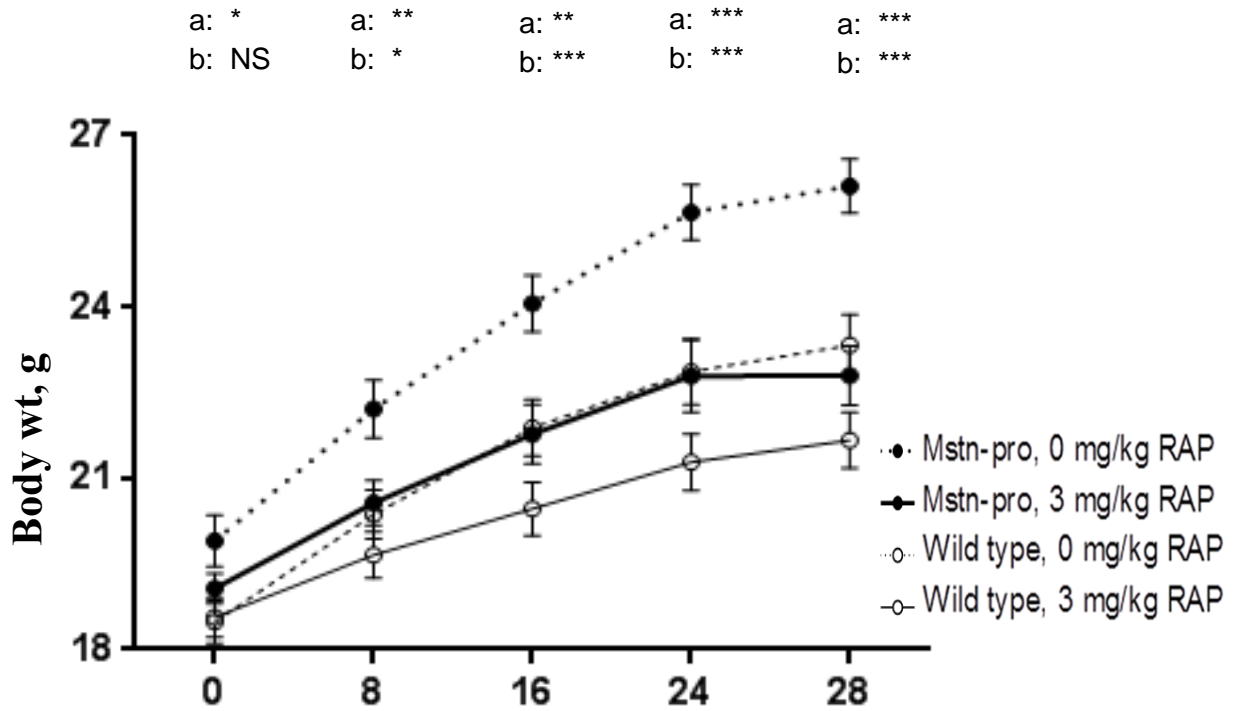


Figure 2.1. Effect of RAP administration on body weight of wild type and Mstn-pro mice. Solid and dotted lines indicate 3 and 0 mg/kg RAP administration, respectively, to wild type (open circle) and Mstn-pro mice (closed circle). Values are means (\pm SEM). a, difference between genotype; b, difference between RAP administration (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; NS, not significant).

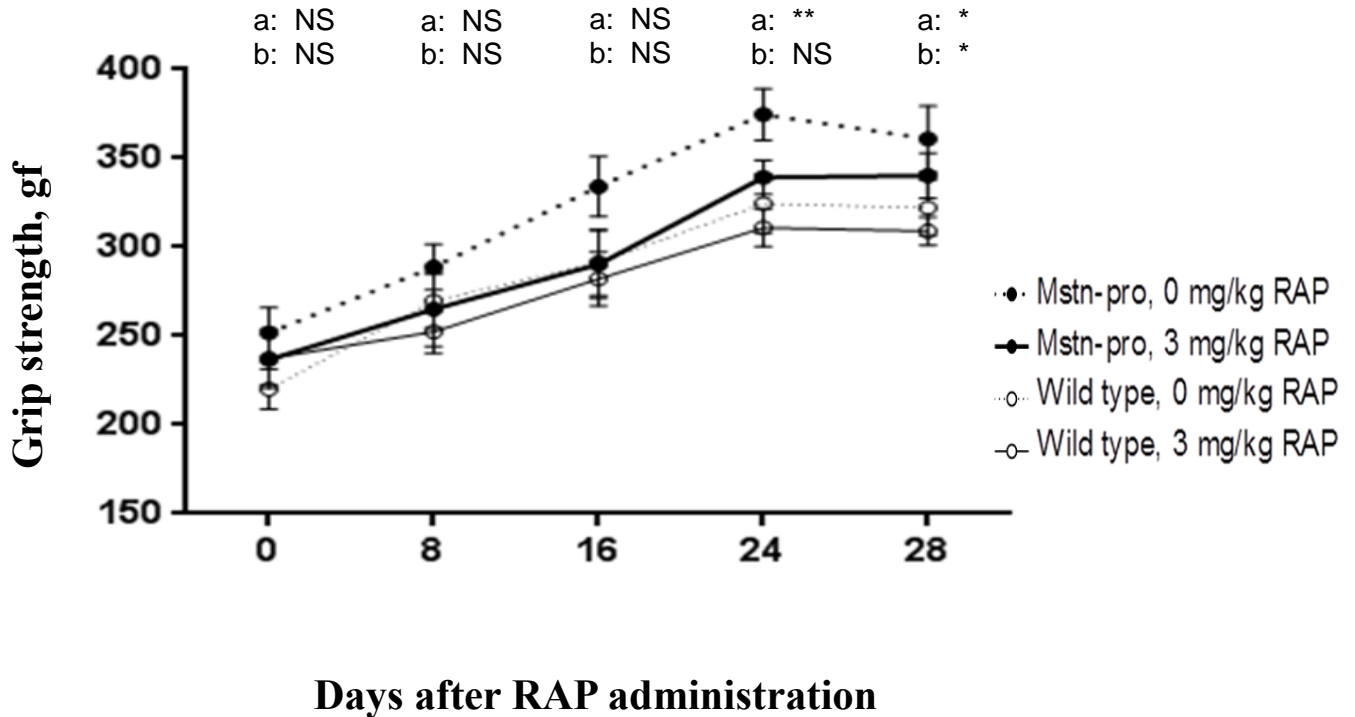


Figure 2.2. Effect of RAP administration on grip strength of wild type and Mstn-pro mice. Solid and dotted lines indicate 3 mg/kg and 0 mg/kg RAP administration, respectively, to wild type (open circle) and Mstn-pro mice (closed circle). Values are means (\pm SEM). a, difference between genotype; b, difference between 0 mg/kg and 3 mg/kg RAP administration (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; NS, not significant). gf, gram force.

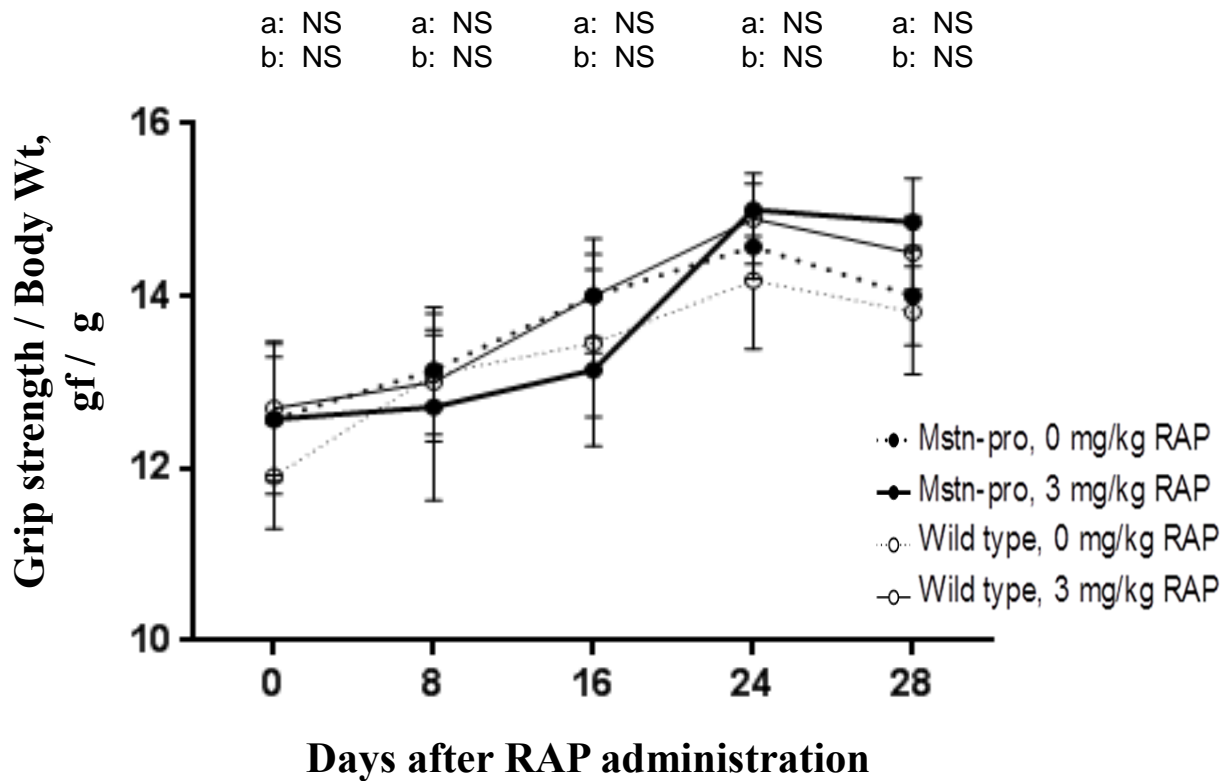


Figure 2.3. Effect of RAP administration on grip strength/ body weight in wild type and Mstn-pro mice. Solid and dotted lines indicate 3 mg/kg and 0 mg/kg RAP administration, respectively, to wild type (open circle) and Mstn-pro mice (closed circle). Values are means \pm SEM. Grip (a, difference between genotype: b, difference between 0 mg/kg and 3 mg/kg RAP administration (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; NS, not significant). gf, gram force.

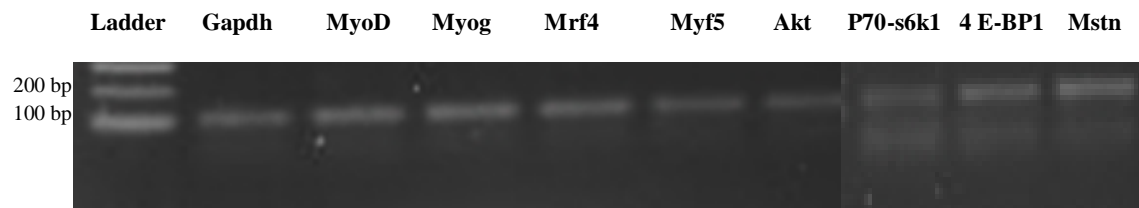
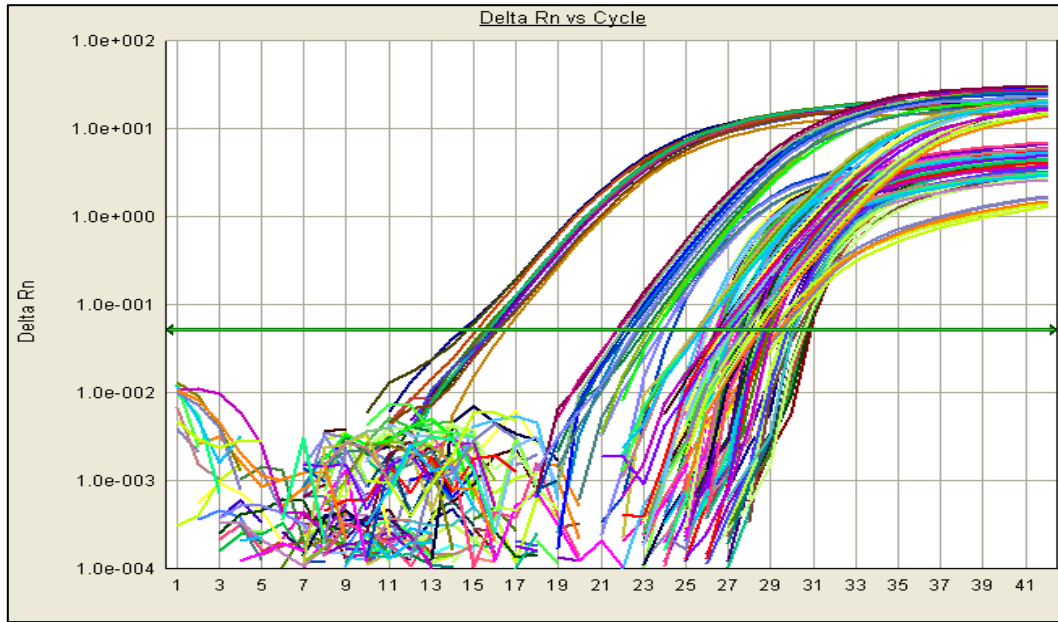


Figure 2.4. PCR products of various genes with the primer sets in Table 1.

A)



B)

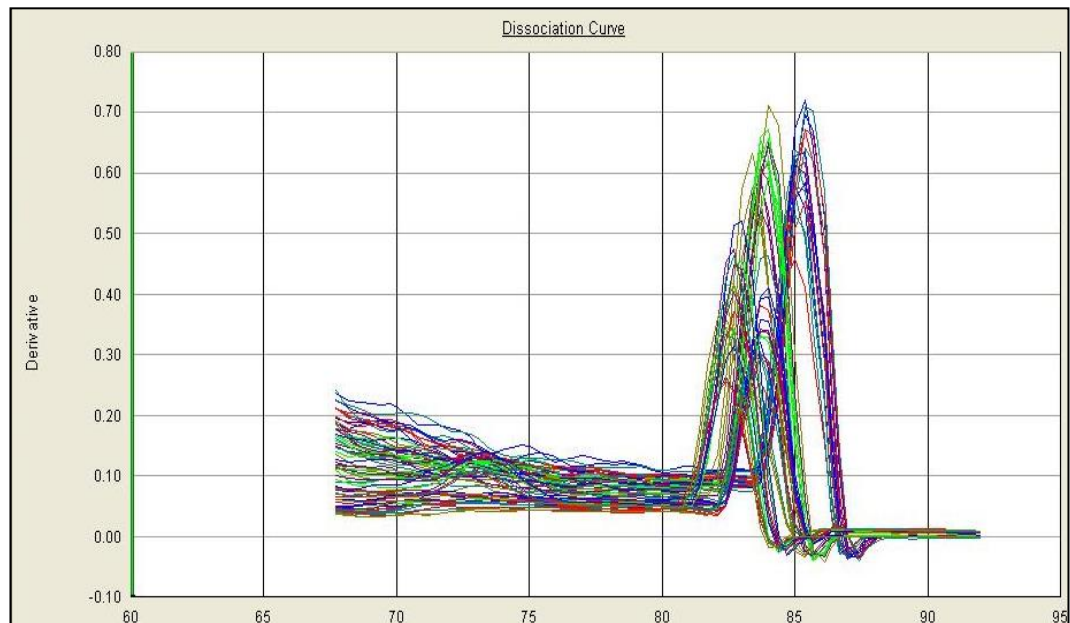


Fig 2.5. Amplification plots and dissociation curve of real-time PCR of all genes

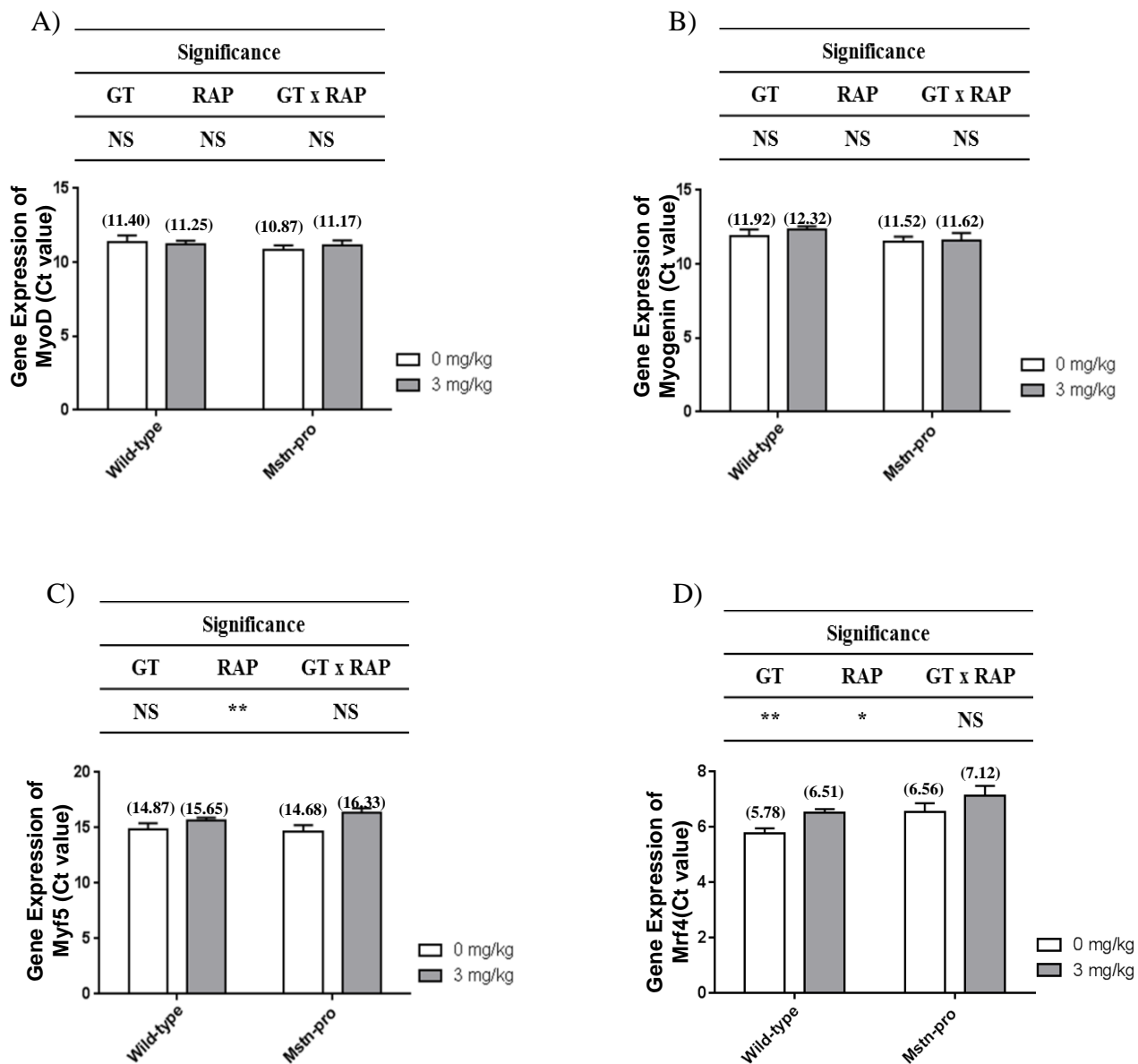


Fig. 2.6. Effect of RAP administration on Myogenic regulatory factors (MyoD, Myogenin, Myf5, Mrf4) expression. Values are expressed as mean \pm SEM. NS, not significant (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; NS, not significance). GT, genotype; RAP, rapamycin

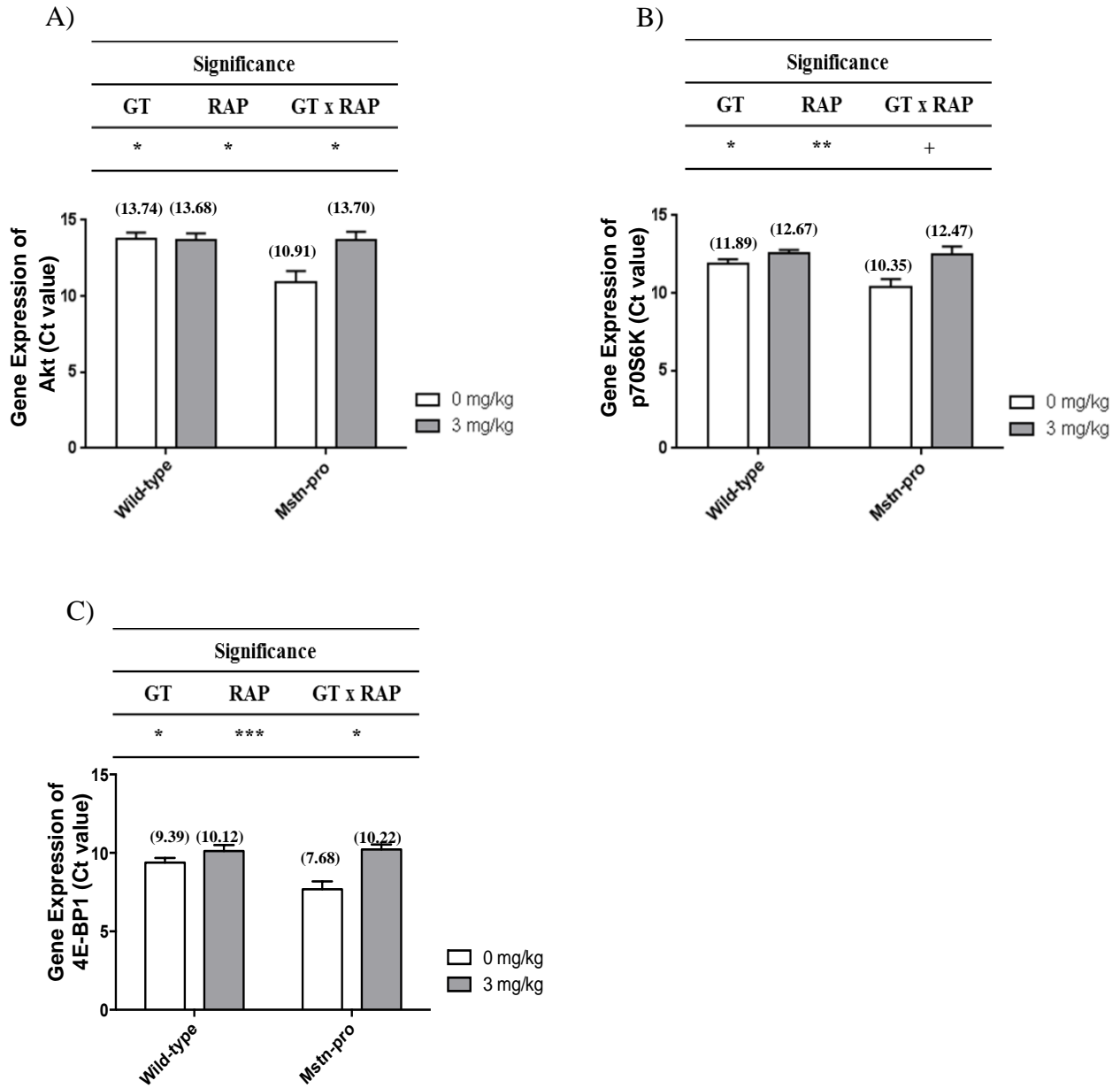


Fig 2.7. Effect of RAP administration on Akt, p70S6K1, 4E-BP1 expression. Values are expressed as mean \pm SEM. NS, not significant (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; NS, not significance). GT, genotype; RAP, rapamycin

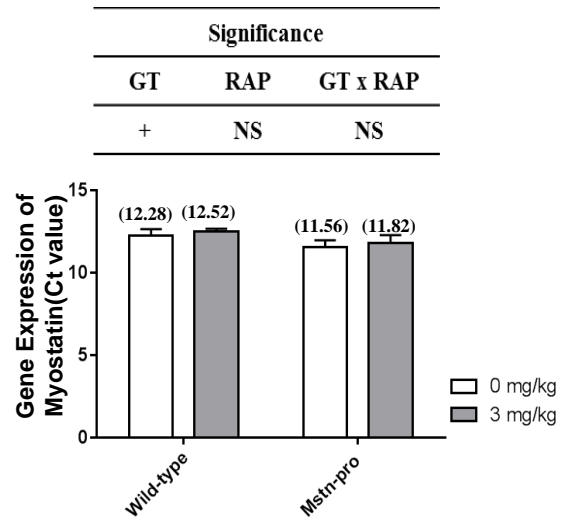


Fig 2.8. Effect of RAP administration on myostatin expression. Values are expressed as mean GT, genotype; RAP, rapamycin; NS, not significant (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; NS, not significance).

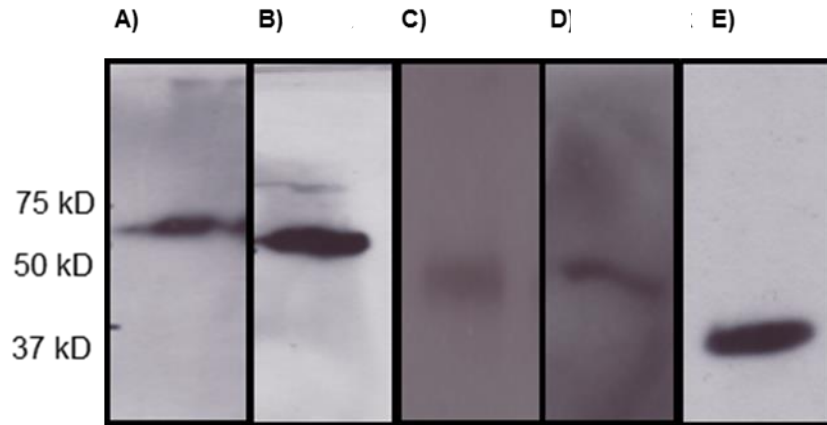


Fig 2.9. Binding of various primary antibodies to fractionated proteins from muscle samples. Membranes were incubated with A) P70S6k, B) P-P70S6k, C) Akt, D) P-Akt, and E) GAPDH primary antibodies. Secondary anti-rabbit antibody IgG was added at 1:10,000 dilution.

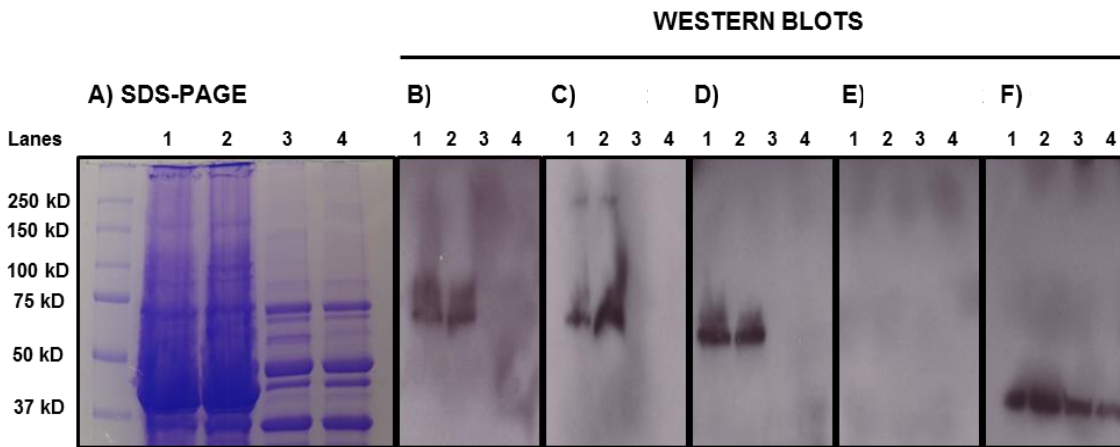


Fig 2.10. Effect of sample storage and effect on antibody binding. Newly prepared samples (lanes 1 and 2), supernatants of freezer stored ground samples (lane 3 and 4). The membranes were incubated with B) P70S6k, C) P-P70S6k, D) Akt, E) P-Akt, F) GAPDH. Secondary anti-rabbit antibody IgG was added at 1:10,000 dilution.

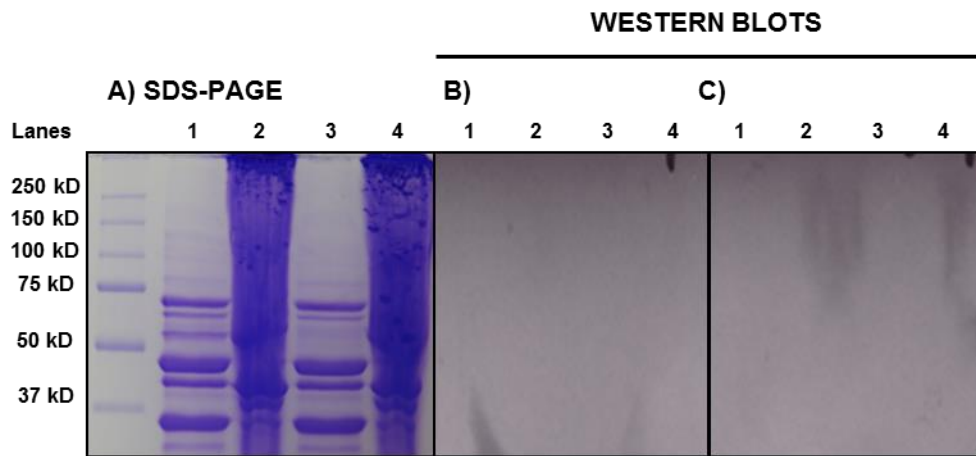


Fig 2.11. Effect of freezer storage of ground samples on immunoblot. Supernatant of freezer stored ground samples (lanes 1 and 3), whole homogenates of freezer stored ground samples (lanes 2 and 4). The membranes were incubated with B) Akt, C) P-Akt antibodies, respectively. Secondary anti-rabbit antibody IgG was added at 1:10,000 dilution

References

- Adel Amirouche, Anne-Cécile Durieux, Sébastien Banzet, Nathalie Koulmann, Régis Bonnefoy, Catherine Mouret, Xavier Bigard, André Peinnequin, and Damien Freyssenet. 2009. Down-Regulation of Akt/Mammalian Target Rapamycin Signaling Pathway in Response to Myostatin Overexpression in Skeletal Muscle. *Endocrinology* 2009 150:1, 286-294
- Amthor H, Nicholas G, McKinnell I, Kemp CF, Sharma M, Kambadur R, Patel K. 2004. Follistatin complexes Myostatin and antagonises Myostatin-mediated inhibition of myogenesis. *Developmental biology* 270:19-30.
- Arsham AM, Howell JJ, Simon MC. 2003. A novel hypoxia-inducible factor-independent hypoxic response regulating mammalian target of rapamycin and its targets. *The Journal of biological chemistry* 278:29655-29660.
- Baar K & Esser K (1999). Phosphorylation of p70(S6k) correlates with increased skeletal muscle mass following resistance exercise. *Am J Physiol Cell Physiol* 276, C120–C127.
- Bentzinger CF, Romanino K, Cloetta D, Lin S, Mascarenhas JB, Oliveri F, Xia J, Casanova E, Costa CF, Brink M, Zorzato F, Hall MN, Ruegg MA. 2008. Skeletal muscle-specific ablation of raptor, but not of rictor, causes metabolic changes and results in muscle dystrophy. *Cell metabolism* 8:411-424.
- Bernardi R, Guernah I, Jin D, Grisendi S, Alimonti A, Teruya-Feldstein J, Cordon-Cardo C, Simon MC, Rafii S, Pandolfi PP. 2006. PML inhibits HIF-1alpha translation and neoangiogenesis through repression of mTOR. *Nature* 442:779-785.
- Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, Bauerlein R, Zlotchenko E, Scrimgeour A, Lawrence JC, Glass DJ, Yancopoulos GD. 2001. Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. *Nat Cell Biol* 3:1014-1019.
- Bogdanovich S, Krag TO, Barton ER, Morris LD, Whittemore LA, Ahima RS, Khurana TS. 2002. Functional improvement of dystrophic muscle by myostatin blockade. *Nature* 420:418-421.
- Brownson, C., & Loughna, P. T. 1996. Alterations in the mRNA levels of two metabolic enzymes in rat skeletal muscle during stretch-induced hypertrophy and disuse atrophy. *Pflügers Archiv*, 431: 990-992.
- Brugarolas J, Lei K, Hurley RL, Manning BD, Reiling JH, Hafen E, Witters LA, Ellisen LW, Kaelin WG, Jr. 2004. Regulation of mTOR function in response to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor complex. *Genes & development* 18:2893-2904.
- Calnan DR, Brunet A. 2008. The FoxO code. *Oncogene* 27:2276-2288.
- Carlson CJ, Booth FW, Gordon SE. 1999. Skeletal muscle myostatin mRNA expression is fiber-type specific and increases during hindlimb unloading. *Am J Physiol* 277:R601-606.
- Clop A, Marcq F, Takeda H, Pirottin D, Tordoir X, Bibe B, Bouix J, Caiment F, Elsen JM, Eychenne F, Larzul C, Laville E, Meish F, Milenkovic D, Tobin J, Charlier C, Georges M. 2006. A mutation creating a

potential illegitimate microRNA target site in the myostatin gene affects muscularity in sheep. *Nat Genet* 38:813-818.

Coleman ME, DeMayo F, Yin KC, Lee HM, Geske R, Montgomery C, Schwartz RJ. 1995. Myogenic vector expression of insulin-like growth factor I stimulates muscle cell differentiation and myofiber hypertrophy in transgenic mice. *The Journal of biological chemistry* 270:12109-12116.

Craig McFarlane, Erin Plummer, Mark Thomas, Alex Hennebry, Murray Ashby, Nicholas Ling, Heather Smith, Mridula Sharma and Ravi Kambadur. 2006. Myostatin induces cachexia by activating the ubiquitin proteolytic system through an NF- κ B-independent, FoxO1-dependent mechanism

Deblon, N., Bourgoin, L., Veyrat-Durebex, C., Peyrou, M., Vinciguerra, M., Caillon, A., Maeder, C., Fournier, M., Montet, X., Rohner-Jeanrenaud, F. and Foti, M. 2012. Chronic mTOR inhibition by rapamycin induces muscle insulin resistance despite weight loss in rats. *British Journal of Pharmacology*, 165: 2325–2340. *J. Cell. Physiol.*, 209: 501–514.

DeYoung MP, Horak P, Sofer A, Sgroi D, Ellisen LW. 2008. Hypoxia regulates TSC1/2-mTOR signaling and tumor suppression through REDD1-mediated 14-3-3 shuttling. *Genes & development* 22:239-251.

Dreyer HC, Fujita S, Cadenas JG, Chinkes DL, Volpi E, Rasmussen BB. 2006. Resistance exercise increases AMPK activity and reduces 4E-BP1 phosphorylation and protein synthesis in human skeletal muscle. *The Journal of physiology* 576:613-624.

Robert A. Frost , Charles H. Lang. 2011. mTor Signaling in Skeletal Muscle During Sepsis and Inflammation: Where Does It All Go Wrong? *Physiology* 26:83-96.

Frias MA, Thoreen CC, Jaffe JD, Schroder W, Sculley T, Carr SA, Sabatini DM. 2006. mSin1 is necessary for Akt/PKB phosphorylation, and its isoforms define three distinct mTORC2s. *Current biology* : CB 16:1865-1870.

Garcia-Martinez JM, Alessi DR. 2008. mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1). *The Biochemical journal* 416:375-385.

Glass DJ. 2005. Skeletal muscle hypertrophy and atrophy signaling pathways. *The international journal of biochemistry & cell biology* 37:1974-1984.

Glover EI, Phillips SM, Oates BR, Tang JE, Tarnopolsky MA, Selby A, Smith K, Rennie MJ. 2008. Immobilization induces anabolic resistance in human myofibrillar protein synthesis with low and high dose amino acid infusion. *J Physiol* 586: 6049–6.

Greer EL, Brunet A. 2008. FOXO transcription factors in ageing and cancer. *Acta Physiol (Oxf)* 192:19-28.

Grobet L, Pirottin D, Farnir F, Poncelet D, Royo LJ, Brouwers B, Christians E, Desmecht D, Coignoul F, Kahn R, Georges M. 2003. Modulating skeletal muscle mass by postnatal, muscle-specific inactivation of the myostatin gene. *Genesis* 35:227-238.

- Grobet L, Poncelet D, Royo LJ, Brouwers B, Pirottin D, Michaux C, Menissier F, Zanotti M, Dunner S, Georges M. 1998. Molecular definition of an allelic series of mutations disrupting the myostatin function and causing double-muscling in cattle. *Mamm Genome* 9:210-213.
- Guertin DA, Sabatini DM. 2007. Defining the role of mTOR in cancer. *Cancer cell* 12:9-22.
- Guertin DA, Stevens DM, Thoreen CC, Burds AA, Kalaany NY, Moffat J, Brown M, Fitzgerald KJ, Sabatini DM. 2006. Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKC α , but not S6K1. *Developmental cell* 11:859-871.
- Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, Turk BE, Shaw RJ. 2008. AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Molecular cell* 30:214-226.
- Hans C, Dreyer , Micah J. Drummond , Bart Pennings , Satoshi Fujita , Erin L. Glynn , David L. Chinkes , Shaheen Dhanani , Elena Volpi , Blake B. Rasmussen. 2008. Leucine-enriched essential amino acid and carbohydrate ingestion following resistance exercise enhances mTOR signaling and protein synthesis in human muscle. *American Journal of Physiology*. 294:392-400.
- Hardie DG. 2007. AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nature reviews Molecular cell biology* 8:774-785.
- Harrington LS, Findlay GM, Lamb RF. 2005. Restraining PI3K: mTOR signalling goes back to the membrane. *Trends in biochemical sciences* 30:35-42.
- Heitman J, Movva NR, Hiestand PC, Hall MN. 1991. FK 506-binding protein proline rotamase is a target for the immunosuppressive agent FK 506 in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences of the United States of America* 88:1948-1952.
- Hitachi K, Nakatani M, Tsuchida K. 2014. Myostatin signaling regulates Akt activity via the regulation of miR-486 expression. *The international journal of biochemistry & cell biology* 47:93-103.
- Hornberger TA, Hunter RB, Kandarian SC, Esser KA. 2001. Regulation of translation factors during hindlimb unloading and denervation of skeletal muscle in rats. *Am J Physiol Cell Physiol* 281:C179-187.
- Hu S, Chen C, Sheng J, Sun Y, Cao X, Qiao J. 2010. Enhanced muscle growth by plasmid-mediated delivery of myostatin propeptide. *J Biomed Biotechnol* 2010:862591.
- Inoki K, Zhu T, Guan KL. 2003. TSC2 mediates cellular energy response to control cell growth and survival. *Cell* 115:577-590.
- Jacinto E, Facchinetti V, Liu D, Soto N, Wei S, Jung SY, Huang Q, Qin J, Su B. 2006. SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. *Cell* 127:125-137.
- Jacinto E, Loewith R, Schmidt A, Lin S, Ruegg MA, Hall A, Hall MN. 2004. Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nature cell biology* 6:1122-1128.

- Jin HJ, Dunn MA, Borthakur D, Kim YS. 2004. Refolding and purification of unprocessed porcine myostatin expressed in *Escherichia coli*. *Protein Expr Purif* 35:1-10.
- Joullia, D., Bernardi, H., Garandel, V., Rabenoelina, F., Vernus, B., & Cabello, G. 2003. Mechanisms involved in the inhibition of myoblast proliferation and differentiation by myostatin. *Experimental cell research* 286: 263-275.
- Kambadur R, Sharma M, Smith TP, Bass JJ. 1997. Mutations in myostatin (GDF8) in double-muscled Belgian Blue and Piedmontese cattle. *Genome Res* 7:910-916.
- Kim E, Goraksha-Hicks P, Li L, Neufeld TP, Guan KL. 2008. Regulation of TORC1 by Rag GTPases in nutrient response. *Nature cell biology* 10:935-945.
- Lalani R, Bhasin S, Byhower F, Tarnuzzer R, Grant M, Shen R, Asa S, Ezzat S, Gonzalez-Cadavid NF. 2000. Myostatin and insulin-like growth factor-I and -II expression in the muscle of rats exposed to the microgravity environment of the NeuroLab space shuttle flight. *J Endocrinol* 167:417-428.
- Langley B, Thomas M, Bishop A, Sharma M, Gilmour S, Kambadur R. 2002. Myostatin inhibits myoblast differentiation by down-regulating MyoD expression. *The Journal of biological chemistry* 277:49831-49840.
- Laplante M, Sabatini DM. 2012. mTOR signaling in growth control and disease. *Cell* 149:274-293.
- Lee SJ, McPherron AC. 2001. Regulation of myostatin activity and muscle growth. *Proc Natl Acad Sci U S A* 98:9306-9311.
- Lee SJ, Reed LA, Davies MV, Girgenrath S, Goad ME, Tomkinson KN, Wright JF, Barker C, Ehrmantraut G, Holmstrom J, Trowell B, Gertz B, Jiang MS, Sebald SM, Matzuk M, Li E, Liang LF, Quattlebaum E, Stotish RL, Wolfman NM. 2005. Regulation of muscle growth by multiple ligands signaling through activin type II receptors. *Proc Natl Acad Sci U S A* 102:18117-18122.
- Lee SJ. 2008. Genetic analysis of the role of proteolysis in the activation of latent myostatin. *Plos One* 3:e1628.
- Lee, S. J., Huynh, T. V., Lee, Y. S., Sebald, S. M., Wilcox-Adelman, S. A., Iwamori, N., ... & Fan, C. M. 2012. Role of satellite cells versus myofibers in muscle hypertrophy induced by inhibition of the myostatin/activin signaling pathway. *Proceedings of the National Academy of Sciences*, 109: E2353-E2360.
- Li Y, Wang Y, Kim E, Beemiller P, Wang CY, Swanson J, You M, Guan KL. 2007. Bnip3 mediates the hypoxia-induced inhibition on mammalian target of rapamycin by interacting with Rheb. *The Journal of biological chemistry* 282:35803-35813.
- Li Z, Zhao B, Kim YS, Hu CY, Yang J. 2010. Administration of a mutated myostatin propeptide to neonatal mice significantly enhances skeletal muscle growth. *Mol Reprod Dev* 77:76-82.

- Lipina C, Kendall H, McPherron AC, Taylor PM, Hundal HS. 2010. Mechanisms involved in the enhancement of mammalian target of rapamycin signalling and hypertrophy in skeletal muscle of myostatin-deficient mice. *FEBS letters* 584:2403-2408.
- Liu CM, Yang Z, Liu CW, Wang R, Tien P, Dale R, Sun LQ. 2008. Myostatin antisense RNA-mediated muscle growth in normal and cancer cachexia mice. *Gene Ther* 15:155-160.
- Liu L, Cash TP, Jones RG, Keith B, Thompson CB, Simon MC. 2006. Hypoxia-induced energy stress regulates mRNA translation and cell growth. *Molecular cell* 21:521-531.
- Lowe, D. A., Lund, T., & Alway, S. E. 1998. Hypertrophy-stimulated myogenic regulatory factor mRNA increases are attenuated in fast muscle of aged quails. *American Journal of Physiology-Cell Physiology*, 275: C155-C162.
- Magee TR, Artaza JN, Ferrini MG, Vernet D, Zuniga FI, Cantini L, Reisz-Porszasz S, Rajfer J, Gonzalez-Cadavid NF. 2006. Myostatin short interfering hairpin RNA gene transfer increases skeletal muscle mass. *J Gene Med* 8:1171-1181.
- Manning BD. 2004. Balancing Akt with S6K: implications for both metabolic diseases and tumorigenesis. *The Journal of cell biology* 167:399-403.
- Manning BD, Cantley LC. 2007. AKT/PKB signaling: navigating downstream. *Cell* 129:1261-1274.
- Matsakas A, Patel K. 2009. Intracellular signalling pathways regulating the adaptation of skeletal muscle to exercise and nutritional changes. *Histology and histopathology* 24:209-222.
- McPherron AC, Lawler AM, Lee SJ. 1997. Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature* 387:83-90.
- McPherron AC, Lee SJ. 1997. Double muscling in cattle due to mutations in the myostatin gene. *Proc Natl Acad Sci U S A* 94:12457-12461.
- Mehra A, Wrana JL. 2002. TGF-beta and the Smad signal transduction pathway. *Biochem Cell Biol* 80:605-622.
- Thomas, M., Langley, B., Berry, C., Sharma, M., Kirk, S., Bass, J., & Kambadur, R. 2000. Myostatin, a negative regulator of muscle growth, functions by inhibiting myoblast proliferation. *Journal of Biological Chemistry* 275: 40235-40243.
- Morine KJ, Bish LT, Pendrak K, Sleeper MM, Barton ER, Sweeney HL. 2010. Systemic Myostatin Inhibition via Liver-Targeted Gene Transfer in Normal and Dystrophic Mice. *Plos One* 5:e9176.
- Morissette MR, Cook SA, Buranasombati C, Rosenberg MA, Rosenzweig A. 2009. Myostatin inhibits IGF-I-induced myotube hypertrophy through Akt. *Am J Physiol Cell Physiol* 297:C1124-1132.
- Mosher DS, Quignon P, Bustamante CD, Sutter NB, Mellersh CS, Parker HG, Ostrander EA. 2007. A mutation in the myostatin gene increases muscle mass and enhances racing performance in heterozygote dogs. *PLoS Genet* 3:e79.

- Perry, R. L., & Rudnick, M. A. 2000. Molecular mechanisms regulating myogenic determination and differentiation. *Front Biosci* 5: D750-767.
- Nader GA. 2005. Molecular determinants of skeletal muscle mass: getting the "AKT" together. *The international journal of biochemistry & cell biology* 37:1985-1996.
- Nobukuni T, Joaquin M, Roccio M, Dann SG, Kim SY, Gulati P, Byfield MP, Backer JM, Natt F, Bos JL, Zwartkuis FJ, Thomas G. 2005. Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. *Proceedings of the National Academy of Sciences of the United States of America* 102:14238-14243.
- Ohanna M, Sobering AK, Lapointe T, Lorenzo L, Praud C, Petroulakis E, Sonenberg N, Kelly PA, Sotiropoulos A, Pende M. 2005. Atrophy of S6K1(-/-) skeletal muscle cells reveals distinct mTOR effectors for cell cycle and size control. *Nature cell biology* 7:286-294.
- O'Neil, T. K., Duffy, L. R., Frey, J. W. and Hornberger, T. A. 2009. The role of phosphoinositide 3-kinase and phosphatidic acid in the regulation of mammalian target of rapamycin following eccentric contractions. *The Journal of Physiology* 587: 3691–3701
- Pallafacchina G, Calabria E, Serrano AL, Kalhovde JM, Schiaffino S. 2002. A protein kinase B-dependent and rapamycin-sensitive pathway controls skeletal muscle growth but not fiber type specification. *Proc Natl Acad Sci U S A* 99:9213-9218.
- Peterson TR, Laplante M, Thoreen CC, Sancak Y, Kang SA, Kuehl WM, Gray NS, Sabatini DM. 2009. DEPTOR is an mTOR inhibitor frequently overexpressed in multiple myeloma cells and required for their survival. *Cell* 137:873-886.
- Pirottin D, Grobet L, Adamantidis A, Farnir F, Herens C, Schroder HD, Georges M. 2005. Transgenic engineering of male-specific muscular hypertrophy. *Proceedings of the National Academy of Sciences of the United States of America* 102:6413-6418.
- Reardon KA, Davis J, Kapsa RM, Choong P, Byrne E. 2001. Myostatin, insulin-like growth factor-1, and leukemia inhibitory factor mRNAs are upregulated in chronic human disuse muscle atrophy. *Muscle Nerve* 24:893-899.
- Rebbapragada A, Benchabane H, Wrana JL, Celeste AJ, Attisano L. 2003. Myostatin signals through a transforming growth factor beta-like signaling pathway to block adipogenesis. *Mol Cell Biol* 23:7230-7242.
- Reiling JH, Hafen E. 2004. The hypoxia-induced paralogs Scylla and Charybdis inhibit growth by down-regulating S6K activity upstream of TSC in *Drosophila*. *Genes & development* 18:2879-2892.
- Ríos, R., Carneiro, I., Arce, V. M., & Devesa, J. 2002. Myostatin is an inhibitor of myogenic differentiation. *American Journal of Physiology-Cell Physiology* 282: 993-999.
- Rommel C, Clarke BA, Zimmermann S, Nunez L, Rossman R, Reid K, Moelling K, Yancopoulos GD, Glass DJ. 1999. Differentiation stage-specific inhibition of the Raf-MEK-ERK pathway by Akt. *Science* 286:1738-1741.

- Sabatini DM, Erdjument-Bromage H, Lui M, Tempst P, Snyder SH. 1994. RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. *Cell* 78:35-43.
- Sancak Y, Peterson TR, Shaul YD, Lindquist RA, Thoreen CC, Bar-Peled L, Sabatini DM. 2008. The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science* 320:1496-1501.
- Sancak Y, Thoreen CC, Peterson TR, Lindquist RA, Kang SA, Spooner E, Carr SA, Sabatini DM. 2007. PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. *Molecular cell* 25:903-915.
- Sarbassov DD, Ali SM, Kim DH, Guertin DA, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM. 2004. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Current biology : CB* 14:1296-1302.
- Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 307:1098-1101.
- Sartori R, Milan G, Patron M, Mammucari C, Blaauw B, Abraham R, Sandri M. 2009. Smad2 and 3 transcription factors control muscle mass in adulthood. *Am J Physiol Cell Physiol* 296:C1248-1257.
- Schuelke M, Wagner KR, Stolz LE, Hubner C, Riebel T, Komen W, Braun T, Tobin JF, Lee SJ. 2004. Myostatin mutation associated with gross muscle hypertrophy in a child. *N Engl J Med* 350:2682-2688.
- Se-Jin Lee. 2004. Regulation of muscle mass by myostatin. *Annual Review of Cell and Developmental Biology* 20: 61-86
- Stephen G. Dann, Anand Selvaraj, George Thomas. 2007. mTOR complex1-S6K1 signaling: at the crossroads of obesity, diabetes and cancer. *Trends in Molecular Medicine* 13: 252-259
- Tang L, Yan Z, Wan Y, Han W, Zhang Y. 2007. Myostatin DNA vaccine increases skeletal muscle mass and endurance in mice. *Muscle Nerve* 36:342-348.
- Thedieck K, Polak P, Kim ML, Molle KD, Cohen A, Jenou P, Arriemerlou C, Hall MN. 2007. PRAS40 and PRR5-like protein are new mTOR interactors that regulate apoptosis. *PloS one* 2:e1217.
- Thies RS, Chen T, Davies MV, Tomkinson KN, Pearson AA, Shakey QA, Wolfman NM. 2001. GDF-8 propeptide binds to GDF-8 and antagonizes biological activity by inhibiting GDF-8 receptor binding. *Growth Factors* 18:251-259.
- Tobias Schmelzle, Michael N Hall 2000. TOR, a central controller of cell growth. *Cell* 103: 253-262
- Trendelenburg AU, Meyer A, Rohner D, Boyle J, Hatakeyama S, Glass DJ. 2009. Myostatin reduces Akt/TORC1/p70S6K signaling, inhibiting myoblast differentiation and myotube size. *Am J Physiol Cell Physiol* 296:C1258-1270.
- Vander Haar E, Lee SI, Bandhakavi S, Griffin TJ, Kim DH. 2007. Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. *Nature cell biology* 9:316-323.
- Wan M, Wu X, Guan KL, Han M, Zhuang Y, Xu T. 2006. Muscle atrophy in transgenic mice expressing a human TSC1 transgene. *FEBS letters* 580:5621-5627.

Wang L, Harris TE, Roth RA, Lawrence JC, Jr. 2007. PRAS40 regulates mTORC1 kinase activity by functioning as a direct inhibitor of substrate binding. *The Journal of biological chemistry* 282:20036-20044.

Wehling M, Cai B, Tidball JG. 2000. Modulation of myostatin expression during modified muscle use. *FASEB J* 14:103-110.

Whittemore LA, Song K, Li X, Aghajanian J, Davies M, Girgenrath S, Hill JJ, Jalenak M, Kelley P, Knight A, Maylor R, O'Hara D, Pearson A, Quazi A, Ryerson S, Tan XY, Tomkinson KN, Veldman GM, Widom A, Wright JF, Wudyka S, Zhao L, Wolfman NM. 2003. Inhibition of myostatin in adult mice increases skeletal muscle mass and strength. *Biochemical and biophysical research communications* 300:965-971.

Winbanks CE¹, Weeks KL, Thomson RE, Sepulveda PV, Beyer C, Qian H, Chen JL, Allen JM, Lancaster GI, Febbraio MA, Harrison CA, McMullen JR, Chamberlain JS, Gregorevic P. 2012. Follistatin-mediated skeletal muscle hypertrophy is regulated by Smad3 and mTOR independently of myostatin. *J Cell Biol.* 197(7):997-1008

Wolfman NM, McPherron AC, Pappano WN, Davies MV, Song K, Tomkinson KN, Wright JF, Zhao L, Sebald SM, Greenspan DS, Lee SJ. 2003. Activation of latent myostatin by the BMP-1/tolloid family of metalloproteinases. *Proc Natl Acad Sci U S A* 100:15842-15846.

Wouters BG, Koritzinsky M. 2008. Hypoxia signalling through mTOR and the unfolded protein response in cancer. *Nature reviews Cancer* 8:851-864.

Wullschleger S, Loewith R, Hall MN. 2006. TOR signaling in growth and metabolism. *Cell* 124:471-484.

Yang J, Ratovitski T, Brady JP, Solomon MB, Wells KD, Wall RJ. 2001. Expression of myostatin pro domain results in muscular transgenic mice. *Mol Reprod Dev* 60:351-361.

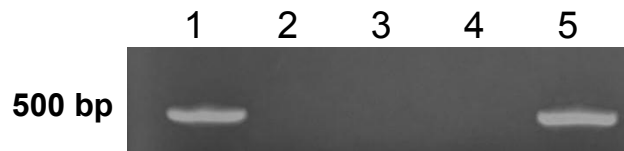
Yimin Fang, Reyhan Westbrook, Cristal Hill, Ravneet K. Boparai, Oge Arum, Adam Spong, Feiya Wang, Martin A. Javors, Jie Chen, Liou Y. Sun, Andrzej Bartke. 2013. Duration of Rapamycin Treatment Has Differential Effects on Metabolism in Mice. *Cell Metabolism* 17: 456-462.

Zhu X, Topouzis S, Liang LF, Stotish RL. 2004. Myostatin signaling through Smad2, Smad3 and Smad4 is regulated by the inhibitory Smad7 by a negative feedback mechanism. *Cytokine* 26:262-272.

Zimmers TA, Davies MV, Koniaris LG, Haynes P, Esquela AF, Tomkinson KN, McPherron AC, Wolfman NM, Lee SJ. 2002. Induction of cachexia in mice by systemically administered myostatin. *Science* 296:1486-1488.

Appendix 2.1

Agarose gel electrophoresis of genotype



1 % agarose gel electrophoresis gel stained with ethidium bromide. Lane 1 and 5, Transgenic mice: Lane 2, 3 and 4 Wild type mice

Appendix 2.2

Mice body weight and muscle raw data.

No	Geno	Treat	0 d	4 d	8 d	12 d	16 d	20 d	24 d	28 d	Sol	Plan	Gas	%Sol	%Plan	%Gas
683	Wild	PBS	17.7	19.1	19.3	20.6	20.7	20.4	21.3	21.6	0.0136	0.0232	0.3008	0.0624	0.1064	1.3798
611	Wild	PBS	19.1	19.9	19.7	20.1	20.6	20.9	21.9	22.4	0.0108	0.028	0.195	0.0489	0.1267	0.8824
597	Wild	PBS	19.3	20.3	21.7	22.5	23.5	23.9	24.5	24.3	0.0178	0.035	0.2056	0.0856	0.1683	0.9885
567	Wild	PBS	20	20.7	21.7	22	22.9	23.2	23.8	25.1	0.013	0.0262	0.2356	0.0537	0.1083	0.9736
559	Wild	PBS	16.9	17.7	19.2	19.4	20.4	20.8	21.3	22.3	0.018	0.0314	0.2392	0.0738	0.1287	0.9803
606	Wild	PBS	19.2	19.6	19.5	20	20.7	21.4	21.8	21.6	0.012	0.0224	0.1922	0.0550	0.1028	0.8817
609	Wild	PBS	18	20.1	20.3	21.6	22.8	23.5	23.7	23.9	0.0114	0.0258	0.2262	0.0467	0.1057	0.9270
688	Wild	PBS	18.8	19.2	19.2	19.8	20.8	21.1	21.2	21.5	0.0128	0.0204	0.1992	0.0598	0.0953	0.9308
684	Wild	RAP	19.5	20.6	20.7	21.2	21.2	21.2	21.5	21.4	0.0118	0.0214	0.1902	0.0549	0.0995	0.8847
613	Wild	RAP	19	20.7	20.2	20.8	21	21.4	21.6	22.8	0.012	0.028	0.2274	0.0506	0.1181	0.9595
563	Wild	RAP	18.1	18	19	18.3	18.9	19.3	19.7	20.3	0.011	0.0284	0.1932	0.0550	0.1420	0.9660
680	Wild	RAP	16.7	17.8	17.2	17.7	18.2	18.6	18.9	19.3	0.0106	0.023	0.1756	0.0541	0.1173	0.8959
564	Wild	RAP	17.8	17.7	19	19.4	20.5	21.4	22.1	22.1	0.017	0.0272	0.2214	0.0810	0.1295	1.0543
569	Wild	RAP	20.4	21.4	21.8	22.4	23	23.5	23.8	24	0.0128	0.0272	0.194	0.0554	0.1177	0.8398

561	Wild	RAP	19.2	20.1	20.7	20.4	22	22.5	23.5	24	0.012	0.021	0.3018	0.0543	0.0950	1.3656
686	Wild	RAP	19.4	20.1	20.7	20.3	20.6	21.1	21.7	21.1	0.0106	0.025	0.1886	0.0477	0.1126	0.8495
574	Trans	PBS	21	21.3	22.4	23.2	24.6	25.1	26	27	0.0146	0.0338	0.3066	0.0545	0.1261	1.1440
570	Trans	PBS	19.1	21.1	22.1	22.5	23.7	23.9	24.9	25.7	0.0158	0.0382	0.2908	0.0622	0.1504	1.1449
562	Trans	PBS	17.9	18.4	19.5	20.1	21.3	22.5	23.1	23.5	0.014	0.032	0.2956	0.0622	0.1422	1.3138
608	Trans	PBS	19.3	21.2	21.8	23.4	24.5	25.6	26.8	27.2	0.0162	0.0506	0.394	0.0574	0.1794	1.3972
672	Trans	PBS	20.7	21.6	23.4	23.7	24.5	25.5	25.9	26.5	0.016	0.0456	0.35	0.0590	0.1683	1.2915
626	Trans	PBS	19.5	21.1	22.9	23.2	24.4	25.6	26	26.3	0.0158	0.0428	0.304	0.0598	0.1621	1.1515
679	Trans	RAP	19.4	20.3	20.8	20.7	21.9	22.4	23.6	23.1	0.0174	0.0424	0.311	0.0731	0.1782	1.3067
558	Trans	RAP	19.8	20	20.4	20.1	21.4	21.3	21.8	21.9	0.018	0.0382	0.3006	0.0826	0.1752	1.3789
671	Trans	RAP	18.3	19.3	20.6	22.3	22.9	24.4	24.8	24.7	0.014	0.0554	0.3672	0.0567	0.2243	1.4866
616	Trans	RAP	19.5	22	22.3	22.3	23.8	24	24.5	24.2	0.0146	0.0314	0.2416	0.0603	0.1298	0.9983
571	Trans	RAP	18.8	19	19.3	19.3	19.9	19.8	20.6	20.8	0.0128	0.0274	0.2094	0.0637	0.1363	1.0418
926	Trans	RAP	20.9	21	22	22.3	22	23.1	23.5	24.1	0.0156	0.0404	0.2778	0.0655	0.1697	1.1672

Animals at 4 week. Geno, Genotype; Wild, Wild type; Trans, Transgenic; RAP, Rapamycin; Sol, Soleus; Plan, Plantaris; Gas, Gastrocnemius; 0 d, start of RAP administration; 4 d, 4 day of RAP administration; 8 d, 8 day of RAP administration; 12 d, 12 day of RAP administration; 16 d, 16 day of RAP administration; 20 d, 20 day of RAP administration; 24 d, 24 day of RAP administration; 28 d, 28 day of RAP administration.

Appendix 2.3

Mice organ weight raw data.

No	Geno	Treat	Carcass	Heart	Liver	Spleen	Kidney	Wh fat	Br fat
683	Wild	PBS	8.7	0.1274	1.2916	0.0764	0.4525	0.1429	0.0922
611	Wild	PBS	7.9	0.1225	1.2559	0.0711	0.4147	0.2634	0.0912
597	Wild	PBS	7.9	0.1261	1.3122	0.0716	0.4826	0.2377	0.0837
567	Wild	PBS	9.5	0.1217	1.4157	0.0837	0.4463	0.1751	0.0997
559	Wild	PBS	9.7	0.1283	1.4107	0.0817	0.4575	0.3196	0.0897
606	Wild	PBS	7.5	0.1476	1.5821	0.0775	0.4325	0.2276	0.1296
609	Wild	PBS	9.4	0.1655	1.5296	0.0712	0.4882	0.1417	0.1067
688	Wild	PBS	8.5	0.1023	1.1817	0.0598	0.4617	0.0699	0.0941
684	Wild	RAP	8.2	0.1204	1.3893	0.0737	0.4767	0.2747	0.1099
613	Wild	RAP	9.1	0.1407	1.5292	0.0878	0.4452	0.2981	0.1059
563	Wild	RAP	7.9	0.1084	1.2531	0.0388	0.3956	0.4072	0.1146
680	Wild	RAP	7.6	0.0974	1.3426	0.0373	0.3891	0.0936	0.1121
564	Wild	RAP	8.3	0.1403	1.6173	0.0517	0.4024	0.282	0.0845
569	Wild	RAP	9.6	0.1938	1.5939	0.0418	0.346	0.3578	0.16
561	Wild	RAP	10.2	0.1243	1.636	0.0518	0.4184	0.3262	0.0997
686	Wild	RAP	9.6	0.1443	1.6452	0.0753	0.4823	0.3083	0.0772
574	Trans	PBS	11.8	0.1402	1.2793	0.0634	0.5105	0.3468	0.0987
570	Trans	PBS	11.4	0.1514	1.3887	0.0555	0.4376	0.2084	0.1103
562	Trans	PBS	10.4	0.1222	1.1534	0.0771	0.3836	0.1768	0.116
608	Trans	PBS	12.1	0.1563	1.9141	0.0827	0.4842	0.1117	0.1227
672	Trans	PBS	12.2	0.1302	1.5372	0.0645	0.5275	0.1612	0.0916
626	Trans	PBS	10	0.1453	1.2453	0.0664	0.5142	0.1372	0.1111
679	Trans	RAP	10.5	0.1441	1.4771	0.0542	0.3892	0.1785	0.092
558	Trans	RAP	10.7	0.1363	1.3446	0.0377	0.4404	0.1637	0.0798
671	Trans	RAP	10.4	0.1208	1.5859	0.0678	0.4646	0.1165	0.1215
616	Trans	RAP	8.8	0.1659	1.5178	0.0564	0.5312	0.5494	0.1314
571	Trans	RAP	8	0.0964	1.2692	0.0441	0.4122	0.1556	0.0987
926	Trans	RAP	9	0.1219	1.2307	0.064	0.442	0.3416	0.0852

Animals at 4 week. Geno, Genotype; Wild, Wild type; Trans, Transgenic; RAP, Rapamycin; Wh fat, White fat; Br fat, Brown fat.

Appendix 2.4

Mice grip strength raw data

No	Geno	Treat	0 d	4 d	8 d	12 d	16 d	20 d	24 d	28 d
683	Wild	PBS	256	273	329	311	333	319	354	357
611	Wild	PBS	227	275	262	352	320	332	374	348
597	Wild	PBS	166	149	177	227	180	197	221	279
567	Wild	PBS	202	284	284	259	289	396	331	321
559	Wild	PBS	171	228	238	262	229	312	296	336
606	Wild	PBS	236	276	281	366	322	327	337	359
609	Wild	PBS	232	290	289	309	349	347	380	407
688	Wild	PBS	260	314	291	284	351	374	352	378
684	Wild	RAP	316	289	246	259	308	288	304	297
613	Wild	RAP	292	292	241	288	314	285	321	338
563	Wild	RAP	206	188	207	186	186	201	267	313
680	Wild	RAP	206	275	274	298	297	307	363	325
564	Wild	RAP	156	214	222	297	241	264	280	279
569	Wild	RAP	195	220	258	238	278	291	340	311
561	Wild	RAP	178	206	247	211	288	277	310	309
686	Wild	RAP	300	292	282	291	306	325	307	318
574	Trans	PBS	314	377	311	401	393	392	424	422
570	Trans	PBS	215	246	266	291	299	289	313	348
562	Trans	PBS	266	244	231	261	269	277	351	324
608	Trans	PBS	227	294	295	390	365	350	403	412
672	Trans	PBS	277	279	324	260	324	315	361	364
626	Trans	PBS	210	226	273	310	315	323	364	377
679	Trans	RAP	272	290	318	390	338	345	371	373
558	Trans	RAP	214	228	206	255	240	244	315	309
671	Trans	RAP	202	228	253	307	325	322	375	375
616	Trans	RAP	211	266	270	351	312	348	336	312
571	Trans	RAP	238	240	234	234	225	249	321	313
926	Trans	RAP	211	248	270	351	312	348	336	312

Animals at 4 week. Geno, Genotype; Wild, Wild type; Trans, Transgenic; RAP, Rapamycin; 0 d, start of RAP administration; 4 d, 4 day of RAP administration; 8 d, 8 day of RAP administration; 12 d, 12 day of RAP administration; 16 d, 16 day of RAP administration; 20 d, 20 day of RAP administration; 24 d, 24 day of RAP administration; 28 d, 28 day of RAP administration

Appendix 2.5

The raw data of real-time PCR

	Geno	Treat	Gene	MyoD		Myog		Mrf4		Myf5		Akt		p70S6K		4E-BP1		Mstn	
No	Wild	PBS	Gapdh	Avg Ct	DCt	Avg Ct	DCt	Avg Ct	DCt	Avg Ct	DCt	Avg Ct	DCt	Avg Ct	DCt	Avg Ct	DCt	Avg Ct	DCt
683	Wild	PBS	16.11	26.99	10.89	27.42	11.32	22.02	5.91	29.11	13.00	29.11	13.00	28.76	12.65	25.55	9.44	29.11	13.01
611	Wild	PBS	17.45	31.17	13.73	31.88	14.43	23.32	5.87	30.05	12.60	29.56	12.12	27.59	10.15	25.62	8.18	27.70	10.26
597	Wild	PBS	16.32	28.04	11.72	28.97	12.65	22.27	5.96	32.42	16.10	30.70	14.38	28.42	12.10	26.38	10.06	29.08	12.76
567	Wild	PBS	17.21	27.53	10.32	29.35	12.14	22.83	5.62	32.38	15.17	30.88	13.67	29.25	12.04	25.97	8.76	28.18	10.97
559	Wild	PBS	17.71	28.80	11.09	28.44	10.73	23.22	5.51	31.89	14.18	31.20	13.49	29.38	11.67	26.67	8.95	30.79	13.07
606	Wild	PBS	16.06	27.58	11.51	27.89	11.83	22.81	6.75	33.09	17.02	30.92	14.86	28.41	12.35	26.58	10.51	29.39	13.32
609	Wild	PBS	16.10	28.11	12.02	27.40	11.31	21.09	4.99	31.15	15.06	28.69	12.60	27.69	11.59	25.03	8.94	28.79	12.69
688	Wild	RAP	15.75	25.67	9.91	26.73	10.97	21.35	5.59	31.58	15.83	31.58	15.83	28.35	12.59	26.01	10.25	27.92	12.17
684	Wild	RAP	16.81	27.96	11.15	28.51	11.70	22.87	6.06	31.57	14.76	28.06	11.25	28.92	12.11	27.72	10.90	29.09	12.28
613	Wild	RAP	16.50	28.63	12.13	28.28	11.78	23.04	6.54	31.99	15.49	31.44	14.94	29.32	12.82	27.10	10.60	29.29	12.79
563	Wild	RAP	16.39	28.27	11.88	28.69	12.30	23.07	6.67	32.42	16.03	31.01	14.62	29.94	13.54	26.43	10.04	29.05	12.65
680	Wild	RAP	15.98	26.93	10.95	28.73	12.75	23.11	7.13	32.18	16.20	31.09	15.11	29.80	13.82	26.70	10.72	29.21	13.23
564	Wild	RAP	16.21	26.47	10.26	28.52	12.31	22.95	6.74	32.55	16.33	30.08	13.87	29.00	12.79	26.16	9.94	29.26	13.05
569	Wild	RAP	16.98	27.98	11.00	29.11	12.13	23.69	6.71	31.36	14.38	30.10	13.12	29.50	12.52	26.88	9.90	28.86	11.89
561	Wild	RAP	16.29	28.11	11.82	29.96	13.67	22.22	5.93	32.52	16.23	30.00	13.71	28.39	12.10	23.98	7.69	28.24	11.96
686	Trans	PBS	15.94	26.72	10.78	27.90	11.96	22.26	6.32	31.71	15.77	28.78	12.84	30.30	14.36	27.10	11.16	28.30	12.35
574	Trans	PBS	16.38	27.84	11.46	27.84	11.46	22.27	5.89	32.91	16.53	24.50	8.12	24.86	8.48	21.83	5.45	28.49	12.11
570	Trans	PBS	17.59	29.28	11.69	29.28	11.69	23.53	5.94	30.69	13.10	29.82	12.23	28.56	10.97	26.66	9.07	29.19	11.60
562	Trans	PBS	16.41	27.00	10.59	27.00	10.59	23.03	6.62	30.85	14.45	28.28	11.87	27.87	11.47	24.64	8.23	28.41	12.00
608	Trans	PBS	15.39	26.53	11.14	27.44	12.05	22.09	6.70	30.52	15.13	28.23	12.84	27.08	11.69	23.59	8.20	28.03	12.64
672	Trans	PBS	15.34	25.38	10.04	25.90	10.56	21.61	6.27	31.06	15.72	26.24	10.90	25.82	10.48	22.99	7.65	26.81	11.47
626	Trans	RAP	19.20	29.51	10.31	31.95	12.74	27.13	7.93	32.35	13.14	28.70	9.50	28.23	9.03	26.70	7.49	28.90	9.70

679	Trans	RAP	14.74	25.43	10.69	26.55	11.82	22.12	7.38	32.55	17.81	29.64	14.90	28.74	14.00	25.90	11.17	27.91	13.17
558	Trans	RAP	15.86	26.77	10.91	28.35	12.49	23.25	7.39	32.08	16.23	30.56	14.71	29.93	14.07	26.56	10.71	28.78	12.93
671	Trans	RAP	15.54	25.64	10.09	26.79	11.25	21.78	6.24	32.70	17.15	30.04	14.50	27.97	12.43	26.29	10.75	27.27	11.73
616	Trans	RAP	16.22	28.09	11.87	28.06	11.84	22.51	6.29	32.72	16.50	30.25	14.02	28.26	12.03	25.79	9.57	28.37	12.15
571	Trans	RAP	16.98	29.17	12.19	29.85	12.87	23.73	6.75	32.12	15.13	29.33	12.34	27.83	10.85	26.14	9.16	27.09	10.11
926	Trans	RAP	15.96	27.22	11.26	25.43	9.47	24.64	8.68	31.09	15.13	27.71	11.75	27.38	11.42	25.91	9.94	26.80	10.84

Animals at 4 week. Geno, Genotype; Wild, Wild type; Trans, Transgenic; RAP, Rapamycin.