MYOSTATIN REGULATION OF THE INSULIN-LIKE GROWTH FACTOR AXIS

By

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MYOSTATIN REGULATION OF THE INSULIN-LIKE

GROWTH FACTOR AXIS

Abstract

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Myostatin is a dynamic inhibitor of skeletal muscle growth in mammals, whose actions are largely attributed to autocrine effects. Recent studies have suggested that myostatin, which is produced in cardiac and skeletal muscle, has additional endocrine interactions due to its presence in circulation. While limited myostatin inhibition of the IGF axis in muscle has been characterized, systemic involvement with the IGF/IGFBP axis is unknown. We measured muscle, heart and liver transcript levels using qRT-PCR in neonate and adult wild type and *mstn* -/- animals. Neonatal skeletal muscle IGF1R and IGFBP-3 mRNA levels were elevated in *mstn* -/- animals. In adult gastrocnemius IGF1R and IGFBP-5 were elevated while those of IGF-I decreased while pectoralis IGFBP-3 levels were reduced. IGF1R and IGF-II mRNA levels were elevated in neonatal mstn -/- hearts, while adult hearts had a decrease in IGF1R. Myostatin regulation of the IGF axis in striated muscle is fiber type specific and reflects the state of tissue development. Increased IGF1R in all neonatal tissues may reflect enhanced IGF responsiveness and clarify why some *mstn* -/- muscles grow better than others. Levels of IGFR, IGFBP-3 and IGFBP-5 mRNA were all elevated in neonatal *mstn-/-* livers and IGF-I and IGFBP-3 levels were elevated in adults. Circulating levels of IGF-I were elevated in adults while

IGFBP-2 were reduced. Myostatin affects endocrine production of liver IGF axis constituents, these constituents being responsible for mediating organismal growth and development. Additionally, the ratio of IGF binding proteins to IGF dropped, likely increasing IGF bioavailability and contributing to *mstn* knockout animal's double muscled phenotype. Additional experiments with recombinant glycosylated IGFBP-3 mutants characterized IGF -dependent and -independent effects on myoblast proliferation determined that IGF-dependent effects were largely responsible. These data together suggest that myostatin's influence on striated muscle growth includes local and systemic regulation of the IGF/IGFBP axis and that myostatin-induced changes in the muscle expression and circulating levels of IGFBPs likely influence muscle growth in an IGF-dependent fashion.

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Dedication

This thesis is dedicated to my one true muse, the volatilis preteritus monasteriense, and to the PCA3 gene, which has always been with me, and will take care of me in my old age

Chapter One

INTRODUCTION

Myostatin Regulation of Muscle Mass

Myostatin is a member of the transforming growth factor (TGF) -β superfamily and known muscle growth inhibitor in mammals (Rodgers and Garikipati 2008). Originally labeled growth and differentiation factor 8 (GDF-8) when first discovered in 1997 by McPherron *et al*., myostatin was discovered while screening conserved sequences of the TGF-β superfamily (Kollias and McDermott 2008). Upon identification of the novel sequence, McPherron and Lee generated a myostatin knock-out mouse, which had skeletal muscles two to three times larger than background C57 Bl/6 mice, indicating myostatin's role as a muscle growth inhibitor (McPherron, Lawler et al. 1997). Increased growth was the result of both increased muscle cell number (hyperplasia) and size (hypertrophy), indicating a role in regulating fundamental characteristics of muscle growth and development. After initial studies in mice, animals with myostatin loss of function (LOF) were characterized in bovine, canine, and sheep breeds known for enhanced musculature.

The first double muscled animal with a recognized myostatin mutation was the Belgium Blue, a breed of cattle who have muscles approximately 20% larger than other breeds, the result of an eleven base pair deletion in the *mstn* coding sequences (McPherron and Lee 1997). The resultant peptide is truncated by a premature stop codon which precludes transcription of the majority of the bioactive carboxy-terminal domain (Grobet, Martin et al. 1997). Marchigiana cattle from Italy have similar musculature as the result of a guanine to thymine transversion that introduces an early stop codon into

the myostatin gene and removes the last 254 bases of the third exon (Marchitelli, Savarese et al. 2003). Piedmontese cattle also have a mutation in the third exon of *mstn*, as a cysteine to tyrosine mutation impedes cysteine knot formation, likely inactivating the bioactive peptide (Kambadur, Sharma et al. 1997).

A double muscled phenotype is seen in the bully whippet, a dog breed whose myostatin gene has a 2 base-pair deletion, which removes a critical cysteine residue necessary for forming disulfide bridges in the functional myostatin dimer and likely inactivating mature myostatin (Mosher, Quignon et al. 2007). The effect of myostatin LOF was highly correlated with skeletal muscle size and accounted for 60% of variation in mass to height ration and neck girth as well as 31% of the variation in chest girth (See Figure 1). Texel sheep, which have long been prized for their musculature, have a Guanine to adenine transition in the *mstn* 3` untranslated region (UTR) that allows binding of the miRNAs mir1 and mir206 which reduces myostatin expression and likely results in enhanced musculature (Clop, Marcq et al. 2006). In addition to animal models, a myostatin null phenotype was also described in a young boy (Schuelke, Wagner et al. 2004). No pathological symptoms have been described, although he was capable of holding two 3-kg dumbbells in horizontal suspension with his arms extended at 4.5 years of age. Upon closer investigation, a guanine to adenine mutation was found in the *mstn* gene's first splice site, which introduces a 108-bp intron into the coding sequence, resulting in a premature stop and the translation of a non-functional peptide (Schuelke, Wagner et al. 2004).

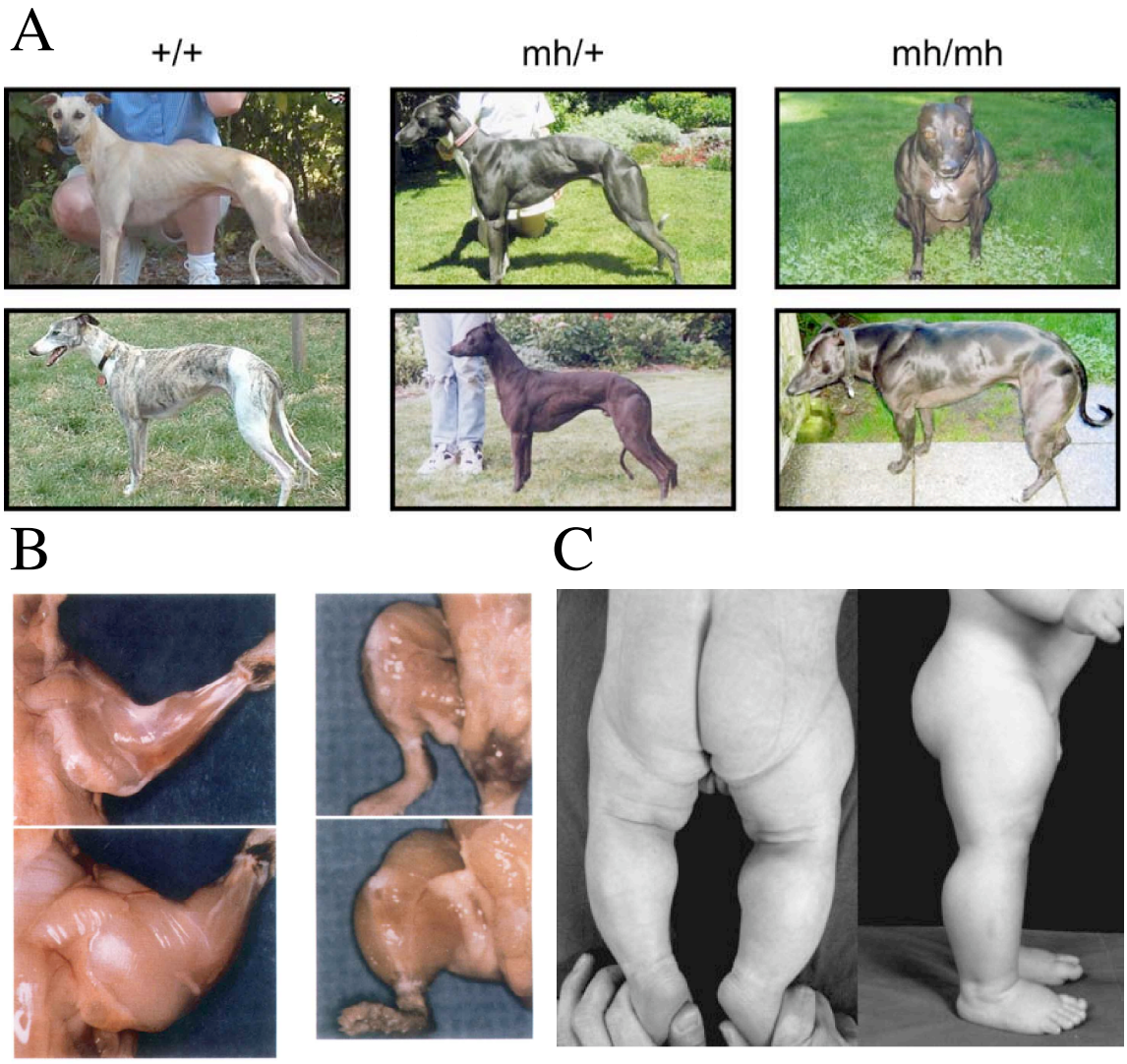

Figure 1. Double Muscled phenotypes A. Racing whippets (left), heterozygous *mstn* loss of function (LOF) whippets (center), and "Bully" whippets with double *mstn* LOF (Mosher, Quignon et al. 2007) B. Wild type (top) and myostatin knockout mice (bottom) and background C57 Bl/6 (McPherron, Lawler et al. 1997) C. Musculature of a seven-month old infant lacking functional myostatin (Schuelke, Wagner et al. 2004)

Myostatin Expression

In addition to strong skeletal muscle expression, myostatin is also expressed in mammalian cardiac muscle, particularly in the Purkinje fibers, contractile structures and coronary artery muscle, although levels are lower than in skeletal muscle (McPherron, Lawler et al. 1997; Sharma, Kambadur et al. 1999; McKoy, Bicknell et al. 2007). Myostatin expression following a myocardial infarction increases in the damaged tissue for a period of weeks, potentially inhibiting proper heart repair and remodeling, though some studies suggest this increase may be due to fibroblast expression (Bass, Oldham et al. 1999; Li, Kollias et al. 2008).

Transcription of myostatin is regulated in muscle by E boxes (CANNTG), which are transactivated by the basic helix-loop-helix differentiation factors MyoD, FOXO and SMADs 2,3 and 4 (Spiller, Kambadur et al. 2002; Allen and Unterman 2007). Myostatin is excreted as a dimer from cells into the extracellular space and circulates as an inactive peptide (Lee 2004). Activation is the result of two cleavage events, first, when the signal sequence is removed, putatively by furin, into 27.7 and 12.4 kda peptides, the larger of which is called the latent associated peptide (LAP) which remains associated with the bioactive portion (Zhu, Hadhazy et al. 2000; Thies, Chen et al. 2001). The second cleavage is regulated by an unknown enzyme, likely a metalloprotease, and serves to produce bioactive myostatin through proteolytic cleavage of LAP (Huet, Li et al. 2001; Lee 2004).

Myostatin Inhibitors and Signaling

A diverse variety of factors bind myostatin and other TGF-β members in circulation and locally. These include follistatin (FS), follistatin related gene (FLRG),

Titin-cap and Growth and differentiation factor associated serum protein 1 (GASP-1) (Hill, Davies et al. 2002; Nicholas, Thomas et al. 2002; Hill, Qiu et al. 2003). *In vitro* assays have characterized titin-cap binding of myostatin, interaction kinetics and regulation of myostatin secretion which blocks its action in muscle cells (Nicholas, Thomas et al. 2002). GASP-1 has been immunoprecipitated with myostatin and used in binding assays to inhibit myostatin action (Hill, Qiu et al. 2003). Furthermore, when GASP-1 is over-expressed in mice, body weight increased in proportion to the reduction in myostatin activity (Haidet, Rizo et al. 2008). *In vitro* studies also identified FLRG binding myostatin, and over-expression results in muscular dystrophy, though to a lesser extent than FS (Hill, Davies et al. 2002; Haidet, Rizo et al. 2008).

Transgenic mice have been used to study follistatin action on muscle, and while follistatin knockout results in death shortly after birth, these animals display reduced muscle mass in intercostal and diaphragm musculature (Matzuk, Lu et al. 1995). Further studies, using mice over-expressing follistatin, have a dose-dependent phenotype similar to *mstn* knockout with up to a 327% increase in musculature, with the well characterized hypertrophy and hyperplasia (Matzuk, Lu et al. 1995; Lee 2007). The use of soluble activin receptor type IIB (ActrIIB), which binds and inactivates myostatin, and FS to improve muscle growth beyond that seen in myostatin null mice suggests the involvement of additional factors, likely members of the highly homologous TGF-β superfamily, in regulating muscle mass (Whittemore, Song et al. 2003; Lee, Reed et al. 2005).

Free myostatin binds primarily to activin receptors ActRIIA and ActRIIB, though with higher affinity for ActRIIB (Lee and McPherron 2001). Activin receptors are serine

and threonine kinases, which form heterotetrameric complexes with activin-like kinase (ALK) -4 and -5 receptors, and phosphorylate SMAD proteins to initiate signaling cascades which regulate transcription (Zhu, Topouzis et al. 2004). Disrupting ALK-4 involvement in the receptor complex abrogates myostatin's action without effecting TGF-β signaling, indicating a critical role in myostatin effect (Harrison, Gray et al. 2004). Once SMAD 2 and 3 are phosphorylated, they dimerize and form a complex with co-SMAD 4, which allows nuclear translocation. The nuclear SMAD 2/3/4 complex acts as a transcription factor to regulate proliferation and differentiation by altering transcriptional patterns and signaling cascades involved in myogenesis (Li, Kollias et al. 2008).

Myostatin Action on Growth and Differentiation

Myogenesis is the formation of muscle fiber and consists of two related but distinct steps: proliferation and differentiation. Muscle proliferation involves stimulation of the mitogen activated protein kinases (MAPK) and Akt/mTOR signaling pathways, which control division and protein synthesis in myosatellite cells (Philippou, Halapas et al. 2007). Myostatin interferes with this process by inhibiting G1 to S-phase transition (Thomas, Langley et al. 2000; Rios, Carneiro et al. 2001; Lee 2004) through decreases of cyclin-dependent kinase 2 (Cdk-2), concurrent increases in p21 transcription and activity, resulting in hypophosphorylation of Rb (Thomas, Langley et al. 2000; Joulia, Bernardi et al. 2003; McCroskery, Thomas et al. 2003). Additionally, myostatin activates p38 through TGF-β-activated kinase 1 (TAK1), inhibiting MAPK's drive of proliferation in a SMAD independent manner (Philip, Lu et al. 2005). Myostatin also down-regulates the Akt/mTOR pathway, which is largely responsible for driving proliferation by

inactivating PI3K, which induces p300 degradation to the detriment of cyclin D1 expression (Morissette, Cook et al. 2006; Yang, Zhang et al. 2007; Ji, Zhang et al. 2008).

Myosatellite cells can also arrest their cell cycle and, under the control of differentiation factors, actively differentiate into myoblasts to maintain, repair, or enlarge muscle fibers (McCroskery, Thomas et al. 2003). Myostatin's role in this process is suspected to either induce cellular quiescence or simply delay differentiation. Experiments studying myosatellite survival, a characteristic seen in quiescent cells, in C2C12 myoblasts demonstrate dose-dependent reduction of differentiation factors MyoD, Myf-5, and to some degree, myogenin (Langley, Thomas et al. 2002; Rios, Carneiro et al. 2002). Additional work by Amthor revealed that under myostatin's influence, MyoD and myogenin expression largely ceases in developing chicken limb buds, but that Myf5 remains sTABLE (Amthor, Otto et al. 2006). However, this inhibiton of factors is reversible and when myostatin is removed from the limb buds, with differentiation factors returning to near normal levels. Further studies with *mstn* -/- satellite cells showed higher satellite cell populations, which are reduced to wild type levels in the presence of myostatin and express differentiation factors in a delayed manner (McCroskery, Thomas et al. 2003; Wagner, Liu et al. 2005). Other experiments with green fluorescent protein tagged myostatin constructs inserted into somites showed that satellite cells producing myostatin and p21 also express MyoD, indicating cell cycle withdrawl for the purpose of differentiation (Manceau, Gros et al. 2008).

Myostatin in the Young and Old

In muscle precursor cells, myostatin inhibits proliferation and inactivates Pax7, a common muscle stem cell marker, via ERK 1/2 signaling pathways. This results in the

depletion of satellite cell populations and decreases both Akt activation and muscle mass (Rios, Carneiro et al. 2002; Amirouche, Durieux et al. 2008; McFarlane, Hennebry et al. 2008). As maturation continues, depletion of myoblast precursors results in reduced muscle mass and impaired muscle growth and development (Manceau, Gros et al. 2008). In young myostatin knockout animals, satellite cell populations are approximately double those found in background mice, although there is little morphology or muscle size change until later in life (McCroskery, Thomas et al. 2003; Morissette, Cook et al. 2006; Siriett, Platt et al. 2006).

As mice, rats and cattle age, myostatin expression in skeletal muscles decreases, although myostatin remains a dynamic factor in regulating muscle mass (Baumann, Ibebunjo et al. 2003; Shibata, Matsumoto et al. 2006; Nishimura, Oyama et al. 2007). *Mstn* -/- and wild type animals maintain satellite cell populations at approximately 11% and 5%, respectively. Disruption of myostatin, as seen with the administration of soluble ActRIIB receptor and α-myostatin antibodies, induce hypertrophy regardless of age (Welle 2002; Whittemore, Song et al. 2003; Siriett, Platt et al. 2006; Welle, Bhatt et al. 2007; Lee, Reed et al. 2005; Bogdanovich, Krag et al. 2002). These results indicate myostatin is intimately involved in regulating proliferation and differentiation of both myoblasts and established muscle by influencing, either directly or indirectly, a diverse array of signaling pathways. One of the first hypothesized pathways, first investigated by Semsarian *et al.* in 1999, was the insulin like growth factor (IGF) axis.

Myostatin and the IGF axis

The IGF axis is primarily a growth hormone (GH) driven system responsible for regulating many aspects of somatic tissue growth (Jones and Clemmons 1995). The axis

consists of IGF -I and –II, six IGF binding proteins (IGFBPs) that modulate IGF bioavailability and activity, and the type 1 IGF receptor (IGF1R), which is found in virtually every tissue of the body (Yan, Forbes et al. 2004; Rodgers, Roalson et al. 2007). GH is secreted from the anterior pituitary, circulates through the body and stimulates liver production of acid labile subunit (ALS) and IGF-I, and in turn IGF-I stimulates IGFBP-3 production (Kelley, Oh et al. 1996; Villafuerte, Zhang et al. 1996). These factors enter the circulation and form a 150 kilo-dalton protease resistant tertiary complex of IGF-I/IGFBP-3/ALS which circulates throughout the body and maintains a reservoir of IGF-I (Jones and Clemmons 1995). Furthermore, IGF constituents are also produced locally and have autocrine and paracrine effects in addition to systemic effects (Adams 2002; Yakar, Pennisi et al. 2005).

Circulating IGF-I levels help regulate GH release through a negative feedback loop (Wallenius, Sjogren et al. 2001). The local versus systemic effects of IGF-I were recently studied with Liver IGF-1 deficient (LID) and acid labile subunit knock out (ALSKO) mice (Yakar, Rosen et al. 2002). After observing that ALSKO and LID mice have 65-75% reductions in systemic IGF-I with 10-20% lower body weights, the authors initially concluded that systemic IGFs have a minor role in regulating growth. However, LID/ALSKO double knockout mice have 85-90% reduction in systemic IGF-I levels and 30 % reduction in body weight, suggesting that systemic IGFs play a vital role in regulating whole organism growth (Yakar, Rosen et al. 2002).

It is generally understood that a wide variety of factors, including IGF-I, estrogen, growth hormone, glucocorticoids, fibroblast growth factors (FGF), and bone morphogenic proteins (BMP) help regulate long bone growth in mammals. Regulation

occurs at the epiphyseal plate (a.k.a. growth plate), which is located between the metaphysis and epiphysis and involves elongation through chondroblast proliferation, differentiation, matrix synthesis, and mineralization (van der Eerden, Karperien et al. 2003). As i*gf-I* knockout and LID/ALSKO mouse long bones were respectively 40% and 20% shorter than control mice and tibia and femur length, growth rate, and bone density were all reduced, IGF-I is understood to be perhaps the most important regulator of bone growth (Yakar, Rosen et al. 2002; Mohan, Richman et al. 2003). Muscle involvement was also analyzed by measuring mRNA levels of IGF-I, GH receptor and type-I IGF receptor (IGF1R) levels in LID/ALSKO mice, and though no quantitative measurements were taken, it was noted that IGF1R levels were elevated.

Circulating IGF-I interacts with IGF1R, which is composed of a heterotetrameric transmembrane glycoprotein that dimerizes, autophosphorylates tyrosine residues and activates a variety of proteins including insulin receptor substrate (IRS)-1, IRS-2 and an SH2-containing protein (SHC) (Danielpour and Song 2006). These signaling molecules mediate proliferation through interactions with the MAPK pathway and hypertrophy and survival through Akt and its downstream effectors (Baxter 2001; Adams 2002; Philippou, Halapas et al. 2007). In addition to stimulating proliferation, IGFs induce differentiation in proliferating myoblasts by activating cyclin-dependent kinase inhibitors and downstream effectors (Lawlor and Rotwein 2000; Fernandez, Dupont et al. 2002). IGF-I has also been found to antagonize myostatin action through the Akt/mTOR pathway and prevent cell death, while at the same time stimulating myostatin transcription (Yang, Zhang et al. 2007). This action may be responsible for governing muscle growth by

maintaining a balance between myocyte proliferation and differentiation and is in accordance with IGF and myostatin action.

Myostatin and the IGFBPs

At the cellular level, IGFs are responsible for regulating both proliferation and differentiation, two processes that are generally antagonistic. One explanation for these contradictory processes may lie in factors that modulate IGF actions, notably IGFBP-3. This is the dominant systemic IGF binding protein as it sequesters approximately 95% of all IGF in circulation. Furthermore, 75% of IGF:IGFBP-3 complexes are bound by ALS in a tertiary complex which increases IGF half life by a factor of 20 (Ma, Pollak et al. 1999). IGFBP-3 is a 27-34 kDa secreted protein produced primarily in the liver in response to IGF-I signaling (Jones and Clemmons 1995). It has three glycosylation states, although no functional significance has been attributed to glycosylation itself (Firth and Baxter 1999). The 42-45 kDa glycosylated proteins contain an N-terminal IGF binding region and a C-terminal metal binding domain (MBD) that also contains a nuclear localization signal (NLS) adjacent to a caveolin box (Jones and Clemmons 1995). This MBD has been implicated in binding interactions with other proteins besides the IGFs (Oufattole, Lin et al. 2006) and appears to be largely responsible for IGFBP-3's IGF-independent effects. IGFBP-3 circulates at high concentrations (2-3 μg/ml) and has long been suspected of having IGF-independent actions on cell growth and differentiation (Diorio, Brisson et al. 2008). IGFBP-3 production is stimulated by TGF-β and mediates some of its effects on cancer cell proliferation, although these effects would appear to be fostering growth (Kansra, Ewton et al. 2000). Some of the traditional roles attributed to the IGFs may in fact be due to IGFBPs, which predominantly work in close conjunction

with IGFs. In recent years, several authors have shown effects independent from the IGFs. Growth inhibitory effects have been well documented in recent years in CHO, L6 and porcine embryonic myoblast cells as well as various prostate and breast cancer cell lines (Lee, Chun et al. 2002; Oufattole, Lin et al. 2006; Ali, Cohen et al. 2003; Pampusch, Kamanga-Sollo et al. 2003; Al-Zahrani, Sandhu et al. 2006; Oufattole, Lin et al. 2006; Xi, Hathaway et al. 2007). Most of the IGF-independent effects of IGFBP-3 involve the inhibition of proliferation or apoptosis through unknown mechanisms. In prostate cancer cells, IGFBP-3 induces apoptosis via a caspase-dependent mechanism despite being unable to bind IGFs (Bhattacharyya, Pechhold et al. 2006). This also occurs in breast cancers as IGFBP-3 reacts synergistically with a ceramide analog to increase apoptosis in proliferating cells (Gill, Perks et al. 1997). Ouffattole *et al*. investigated the effects of IGFBP-3 in myoblasts and after observing IGF-independent inhibition of proliferation, discovered and characterized binding of IGFBP-3 to RNA polymerase II binding subunit 3 (Rpb3), an essential factor for global transcription (Oufattole, Lin et al. 2006). In summary, IGFBP-3 actions involve nuclear translocation of IGFBP-3, binding to available factors, and alteration or repression of transcription, which results in the inhibition of proliferation.

Myostatin involvement with the IGF axis was first investigated with Belgian Blue cattle, where transcription of IGF-II and MyoD were found to be higher (Bass, Oldham et al. 1999). Several years later, investigators used semi-quantitative reverse transcriptase (RT) PCR to characterize expression of IGF constituents in a variety of tissues in *mstn* knock-out mice (Kocamis, Gahr et al. 2002). These studies report IGF1R increases in the heart and kidney and decreases in pectoral muscle, IGF-II decreases in the kidney and no

change in IGF-I. Myostatin expression was also studied in *igf1* knock out mice, but no association was found (Miyake, Hayashi et al. 2007). Finally, clinical studies investigating GH, IGF-I and myostatin association in aged men found that increased GHR levels were correlated to lower myostatin levels, suggesting local myostatin may negatively regulate GHR mRNA levels or that the inverse relationship is regulated by other factors (Marcell, Harman et al. 2001).

Despite the lack of evidence linking myostatin to the IGFs directly, myostatin has been found to have an effect on IGFBP action. Nuclear localization of IGFBP-3 in porcine embroyonic myogenic cells (PEMC) is enhanced when cells are stimulated with TGF-β and myostatin (Xi, Hathaway et al. 2007). Additionally, inactivating IGFBP-3 with antibodies blocks myostatin's inhibition of proliferation in these cells (Pampusch, Kamanga-Sollo et al. 2003). To suggest, however, that myostatin regulates the IGF axis implies more than simple crosstalk in muscle cells. As the IGF axis has both systemic and local action, mediated by receptors in virtually every tissue, any association must also suggest the potential for systemic effects in conjunction with or under the direct regulation of myostatin. The most likely point of cross talk from a physiological perspective is the systemic regulation of liver IGF-I production. Zimmer *et al*. detected myostatin in circulation at 80 ng/ml and increased systemic myostatin by injecting myostatin over-expressing CHO cells into mouse muscles, which led to cachexia and reduced fat deposition (Zimmers, Davies et al. 2002). The fat depositions were reduced due to systemic effects of myostatin and provide a rationale to support further study of circulating myostatin's function. In the liver, which has ActRIIA and ActRIIB receptors, activin induces hepatocyte apoptosis; an effect that can be blocked with dominant

negative mutants of either ActRIIB or Smad2 or stimulated by over-expressing ActRIIB, these being signaling pathways that myostatin shares (Chen, Woodruff et al. 2000; Gold, Francis et al. 2003; Tsuchida 2004). As the liver is the primary site of systemic IGF and IGFBP production, endocrine myostatin has the potential to regulate the IGF and IGFBP axis in the liver.

CHAPTER TWO

MYOSTATIN REGULATION OF THE INSULIN-LIKE GROWTH FACTOR AXIS

Introduction

Studies of myostatin have implicated akt/mTOR, a pathway long understood to be regulated in part by the IGF axis. Investigations of myostatin in IGF-I knockout mice demonstrate no effect on myostatin transcription (Miyake, Hayashi et al. 2007). Previous studies evaluating IGF-axis expression in myostatin knockout mice found few significant differences in mRNA levels, however these studies employed semi-quantitative RT-PCR, which allows for little resolution and gives no information about circulating protein levels (Kocamis, Gahr et al. 2002). The experiments described herein used quantitative RT-PCR to evaluate expression of several IGF axis components, including; IGF-I, IGF1R, IGFBP-3, IGFBP-5, and IGF-II in striated muscle and liver from wild type and myostatin null mice. IGFBP-3 and -5 were analyzed, as their importance in regulating myocytes growth and development are well characterized (Awede, Thissen et al. 1999; Foulstone, Savage et al. 2003). Both neonate and adult mice were analyzed as expression of the IGF axis changes dramatically as mice age, especially during and after puberty. Further examination of systemic IGF axis components was performed in adult mice using ELISAs for direct quantitation of circulating protein levels.

Materials and Methods

Animals. Myostatin knock-out mice were generously donated by Prof. Se-Jin Lee, Johns Hopkins University. Mice were housed and bred in the experimental animal laboratory building, Washington State University. C57 BL/6 J were kindly provided by Dr. Derek McLean, Washington State University. Animals were housed in

environmentally controlled rooms with 12 h daily light and used according to protocols pre-approved by the Institutional Animal Care and Use Committees (IACUC) at WSU.

Quantitative RT-PCR. Total RNA was isolated from the following C57 BL/6 and *mstn* -/- mice: neonatal heart, liver and calf, and seven month old heart, liver, and gastrocnemius and pectoralis muscles. Tissues were homogenized with Trizol (Invitrogen, www.invitrogen.com) according to the manufacturers protocols and RNA was visualized with agarose gel electrophoresis to assess quality. Quantification and purity analysis was assessed with a Spectra Max Plus 384 (Molecular Devices, www.moleculardevices.com) system at 260/280 nm. RNA was treated with DNase I (Ambion Inc.) according to manufacturers protocols before cDNA synthesis from 0.5 μg total RNA with oligo d(t)18 and Invitrogen's SuperScript® III First-Strand Synthesis System. 1 μl (25 ng) of experimental samples was diluted into sample stocks of 9.5 μl dH20, 12.5 μl iQ SYBR Green Supermix before aliquoting in duplicate and addition of 1 μl of both forward and reverse 10 μM primer stock for a final primer concentration of 400 nM for each. GAPDH was used as a reference gene for skeletal muscle and liver while β-actin was used for cardiac tissue, all primers were from previously published papers (Caton, Bringas et al. 2005; Kass, Bridges et al. 2007; Nagata, Masumoto et al. 2007; Kim, Wende et al. 2008). See TABLE 1 for primer sequences. Amplicons were subjected to melt curve analysis and agarose gel electrophoresis to determine specificity and to check for genomic DNA contamination. An iCycler iQ Real-Time PCR Detection System (Bio-Rad, www.biorad.com) was used to amplify samples for 50 cycles of 94°C for 30 s, followed by 55°C for 30 s and 72°C for 30 s.

Quantifying circulating IGF-axis components. ELISAs were performed as described (Hwang, Lee et al. 2008) Microtiter plates were coated with 0.5 μg/well of monoclonal capture antibody in 100 μl of PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, PH 7.4) and incubated overnight at room temperature on a shaker, washed 3 times with 300 μl wash buffer (.05% Tween-20 in PBS). Wells were then incubated with 300 μl blocking buffer (PBS, 5% Tween-20, 5% sucrose, .05% NaN_3) and washed three times with wash buffer. Standards were diluted in assay buffer (50 mM sodium phosphate, 150 mM NaCl, 0.1% Tween-20, .25% BSA, pH 7.4) in concentrations from 0 to 25 ng/mL. Serum samples had 100 μl acid/ethanol reagent (12.5% 2N HCl, 87.5 % ethanol v/v) added followed by 30 minutes incubation at RT before centrifugation at 10,000 rpm for 10 minutes. 50 μl supernatant was neutralized with 25 μl 1M Tris base and diluted with assay buffer 40-100 fold. Samples and control were shaken for 2 hours at room temperature and washed three times before adding 100 μl of a streptavidin-HRP conjugated goat secondary. Samples were then incubated for 20 min. at room temperature and washed four times with before adding 100 μl of OPD solution (1 mg/ml in hydrogen peroxide substrate). After 10 minutes the reaction was stopped by adding 50 μl per well of 2 N H_2SO_4 and fluorescence was quantified using a plate spectrophotometer (490 nm).

Cell culture. L6 rat skeletal myoblasts were cultured at 37°C in 5% CO2 humidified atmosphere in Dulbecco's Modified Eagle's medium (HyClone) supplemented with 10% fetal bovine serum (Atlas Biologicals), 200 mM glutamine, 100 IU/ml penicillin, 0.1 mg/ml streptomycin and 250 ng/ml amphoterecin B. Following plating at 15% confluency, cells were incubated 24 hours, and media was changed to

serum free medium for 2 hours before treatment with recombinant glycosylated IGFBP-3 mutants with and without 4.3 nM IGF-I. After 48 hours incubation, wells were assayed with cell titer 96 proliferation assays (Promega, www.promega.com).

Statistical analysis. Levels of gene expression were quantified using excel qGene method (Muller, Janovjak et al. 2002), followed by analysis with Prism 5 software for OS X. Difference between means was determined with a Student's t-test and ANOVA as appropriate.

Gene Product	Forward/Reverse Primer Sequences (5' - 3')	Temp. (°C)
IGF-I	GGACCAGAGACCCTTTGCGGGG GGCTGCTTTTGTAGGCTTCAGTGG	55
IGF-II	CCTTCGCCTTGTGCTGCAT ACGGTTGGCACGGCTTGAA	55
IGF1R	CTGCGGCGATGAAGAGAAGAAAA TACCGGTGCCACGTTATGATGATT	55
IGFBP-3	AATGGCCGCGGGTTCTGC TTCTGGGTGTCTGTGCTTTGAG	55
IGFBP-5	TGCCTCAACGAAAAGAGCTACG ACAAACTTGGACTGGGTCAGC	55
GAPDH	GACCCCTTCATTGACCTCAAC GATGACCTTGCCCACAGCCTT	55
β-actin	CGCTGCGCTGGTCGTCGACAACG ATCGTACTCCTGCTTGCTGATCCAC	55

TABLE 1. Primer sequences and annealing temperatures. These primer sequences used to amplify amplicons between 150 and 400 bp long for quantitative RT-PCR as discussed in the material and methods.

Results & Discussion

Skeletal muscle mRNA levels of tissue-relevant IGF/IGFBP axis components in wild type and mstn -/- mice.

Neonatal muscle is largely regulated by tissue specific expression of growth factors, primarily IGF-II, whose bio-active functions are largely mediated by IGF1R (Fant and Weisoly 2001). This role was observed in both wild type and myostatin null neonates with strong expression of IGF-II (Figure 2). Expression of IGF1R was also high in wild type and *mstn* -/- neonates, indicating heightened sensitivity towards IGF signaling in neonatal muscle, likely from autocrine/paracrine IGF-II. IGFBP-5 expression was stronger than IGFBP-3, suggesting greater involvement in developing muscle. In adults, expression of IGF-I, IGF1R and IGFBP-3 decreased. Interestingly, IGFBP-5 expression was very similar in neonate and adult mice.

When comparing neonate wild type and *mstn* -/- animals, IGF1R levels were elevated, indicating the potential for IGF signaling in developing myoblast and myocyte populations (Figure 3). Additionally, IGFBP-3 transcripts were increased, and while the actions of non-vascular IGFBPs are context sensitive, IGFBP-3 is locally believed to facilitate IGF binding to IGF1R, resulting in increased growth factor signaling and proliferation (Clemmons 1998). In adults, both gastrocnemius and pectoralis muscle normalized expression changed in a similar manner compared to neonates, however when compared with wild type animals, *mstn* -/- gastrocnemius muscle has elevated IGF1R, which may confer additional IGF sensitivity to this tissue. Pectoralis muscle has decreased IGFBP-3, the significance of which is not known, but may influence IGF receptor interactions.

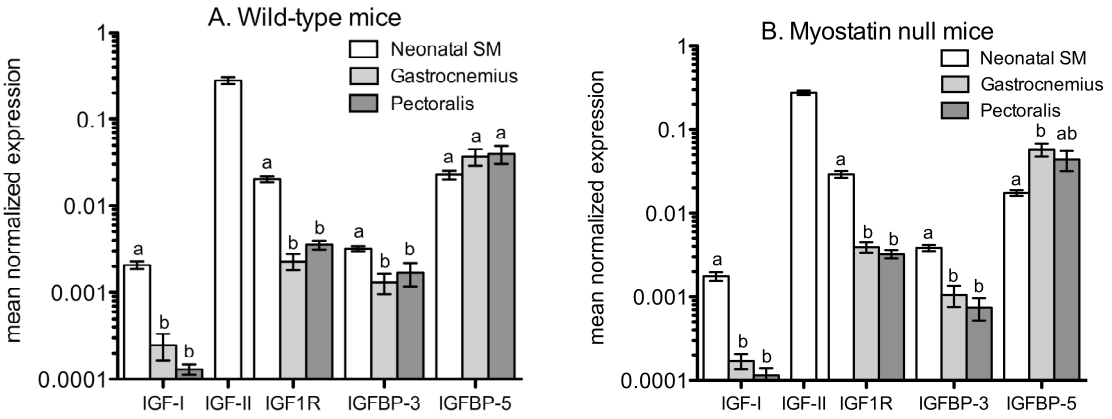

Figure 2. Skeletal muscle mRNA quantification in wild type and *mstn* -/- mice.

Normalized expression values from wild type and myostatin null mice used for quantitative RT-PCR relative to the GADDH reference gene, which is set as 1. Assays were performed at least twice and mean values are for all animals. Error bars represent interassay variation. Letters indicate differences at α=.05 for each gene. (wt n=13, mstn -/- n=11; p=0.05*, 0.01*, 0.001***)

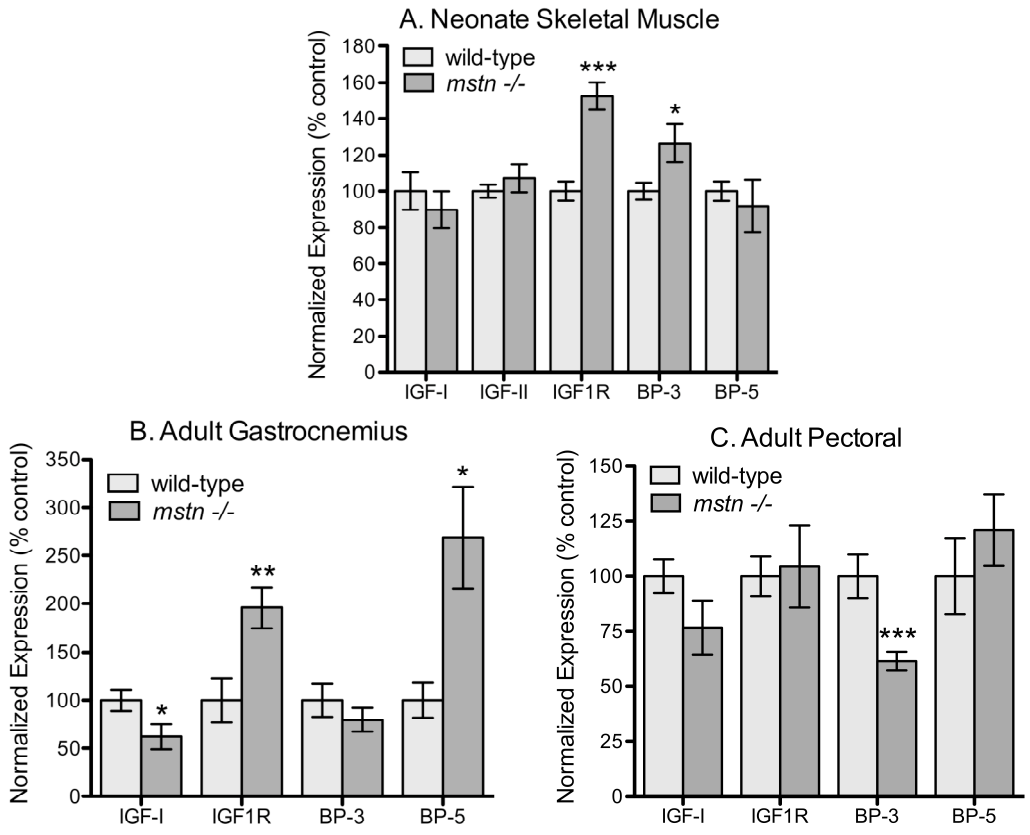

Figure 3. Skeletal muscle mRNA levels of tissue-relevant IGF/IGFBP axis components in wild type and *mstn-/-* mice. Quantitative RT-PCR was performed as described in the materials and methods. Leg skeletal muscle was collected hours after birth and adult tissue from 7 month old mice. Samples were run in duplicate, assays were performed at least twice and GAPDH was used for normalization. Normalized expression values for each gene differed considerably, therefore data was expressed as a % of wild type controls. (wt n=13, mstn -/- n=11; p=0.05*, 0.01*, 0.001***)

% change compared to wild type					
	IGF-I	IGF1R	IGFBP-3	IGFBP-5	IGF-II
Neonatal Muscle	90	153	127	92	107
Neonatal Heart	77	320	154	178	169
Neonatal Liver	56	1244	293	293	82
Gastrocnemius	62	196	80	269	
Pectoral	77	107	63	117	
Adult Heart	66	62	80	96	
Adult Liver	182	77	139	89	
Significant $\alpha \leq .05$					

TABLE 2. Myostatin's effect on IGF axis transcription. Summarized values from all qPCR experiments showing percent change between wild type and myostatin null mice.

The IGF and IGFBP axis in the heart.

Cardiac tissue produces myostatin and growth and proliferation are inhibited in a similar manner to skeletal muscle (Rodgers and Garikipati 2008). However, growth in the heart is mediated in a much different manner than skeletal muscle, with stretching and stress governing rate of growth (Hudlicka and Brown 1996). As in skeletal muscle, IGF-II expression was much greater than IGF-I in neonates, though only ten fold higher (Figure 4). Contrasted with skeletal muscle, IGF1R expression is as strong as IGF-II and indicates the potential for heightened sensitivity to the IGF axis during cardiac development. In adult skeletal muscle, IGF1R and IGFBP-3 are reduced, however IGF-I levels remain the same, suggesting that while IGF-I expression tapers with age in skeletal muscle, its role in cardiac tissue is maintained. Meanwhile, IGFBP-5 drops 250 fold in adults, suggesting that IGFBPs in mature hearts play less of a role in mediating IGF interactions. The effect of *mstn* knockout in neonatal cardiac tissue is increased IGF-II and IGF1R, both of which are likely to drive cardiac proliferation and increase cardiomyocyte populations (Figure 5).

The IGF/IGFBP axis in the liver.

While muscle tissue is strongly influenced by autocrine/paracrine regulation, the liver is the primary source of endocrine IGFs. Systemic factors play a more important role in adults, stimulating organismal growth and maintaining tissue tone. Neonatal liver expression of IGF-I and -II was similar to skeletal muscle, and while an endocrine regulation in neonates is known, developing tissues are largely regulated by the local expression of growth factors (Fant and Weisoly 2001; Nakae, Kido et al. 2001). Other

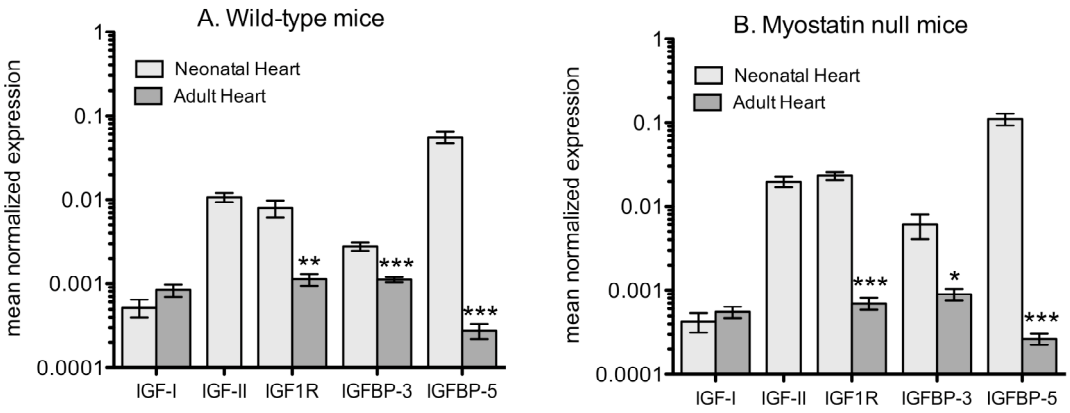

Figure 4. Cardiac muscle mRNA quantification in wild type and *mstn* -/- mice.

Normalized expression values from wild type and myostatin null mice used for quantitative RT-PCR relative to the β-actin reference gene, which is set as 1, otherwise as in Figure2. (wt n=12, mstn -/- n=11); p=0.05*, 0.01*, 0.001***)

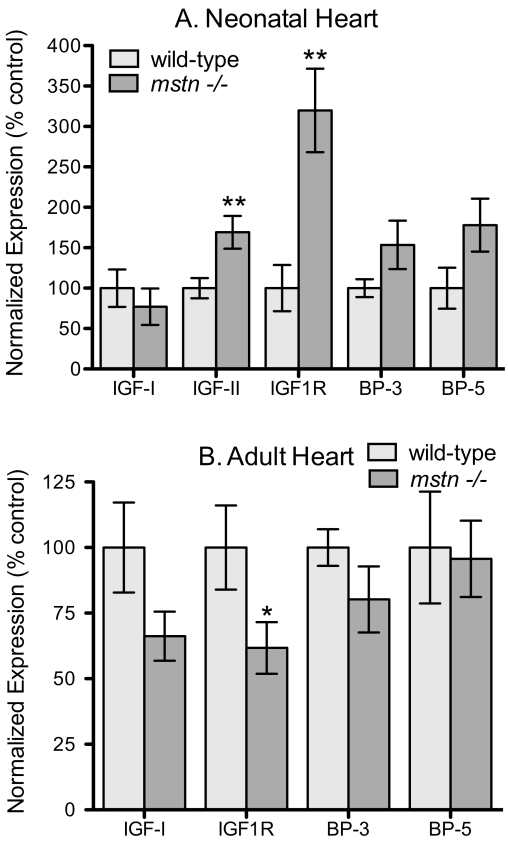

Figure 5. Cardiac muscle mRNA levels of tissue-relevant IGF/IGFBP axis components in wild type and *mstn-/-* mice. Quantitative RT-PCR was performed using β-actin used as reference gene, otherwise as in Figure 3. (wt n=12, mstn -/- n=11); p=0.05*, 0.01*, 0.001***)

IGF axis components were similar to muscle, though IGFBP-5 was much lower suggesting less involvement in IGF interactions at the tissue level (Figure 6).

However, *mstn* knockout dramatically increased IGF1R, IGFBP-3 and IGFBP-5 levels and reduced IGF-I (Figure 7). While IGF-I is reduced, IGF-II is a thousand fold stronger and growth factor stimulation likely remains unchanged. Liver is a secretory organ and IGFs mediate IGFBP secretion, an increase in IGF1R, as seen in all neonatal tissue could well be the cause of IGFBP level elevation.

In adults, liver IGF-I levels increased almost a hundred fold and *mstn* -/- animals had an 82% increase over wild type animals, coupled with increased IGFBP-3, the potential result of IGF stimulation. Liver expression of IGFs is particularly significant in that as the source of approximately 75% of systemic IGF-I, the liver plays and integral role in adults, regulating growth and development (Le Roith, Bondy et al. 2001).

Serum IGF-I levels are elevated.

To further study the increase in IGF-I in adult liver, ELISAs were performed on serum to evaluate IGF-I, IGFBP-1, -2, -3, and ALS levels. Systemic IGF-I increased 49% in knockout animals, reflecting the increase in IGF-I mRNA (Figure 8). The increase in IGF-I stimulation was expected to increase IGFBP-3 levels as well, however, while a slight increase was noted, it was not significant. Interestingly, IGFBP-2, which is primarily regulated by nutrition in a similar manner to IGFBP-1, decreased 29% (Straus 1994). IGFBP-I levels were too low to measure, likely due to the lack of a fasting period, which is necessary to assay IGFBP-1.

Molar ratios of IGF-I to its binding proteins indicated that IGFBP-2, IGFBP-3 and ALS were present in sufficient quantity to bind 39%, 42% and 73%, respectively, in wild

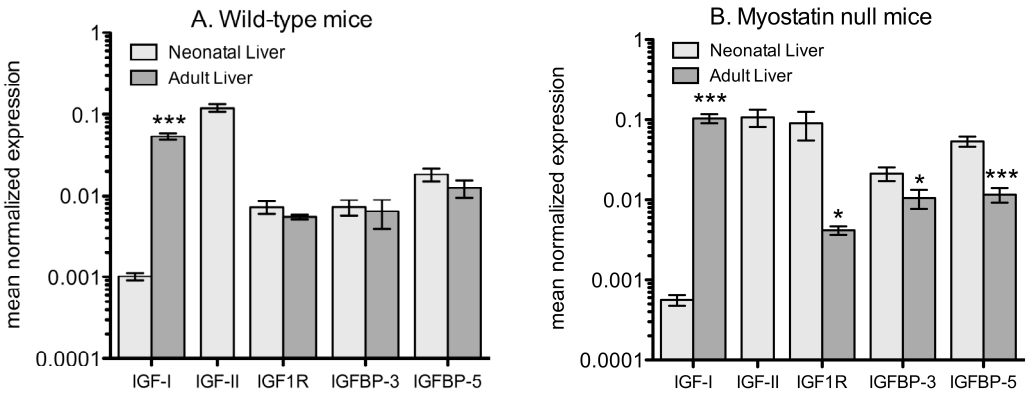

Figure 6. Liver mRNA quantification in wild type and *mstn* -/- mice. Normalized expression values from wild type mice used for quantitative RT-PCR relative to the GAPDH reference gene, which is set as 1, otherwise as in Figure 2. (wt n=12, mstn -/- n=12; p=0.05*, 0.01*, 0.001***)

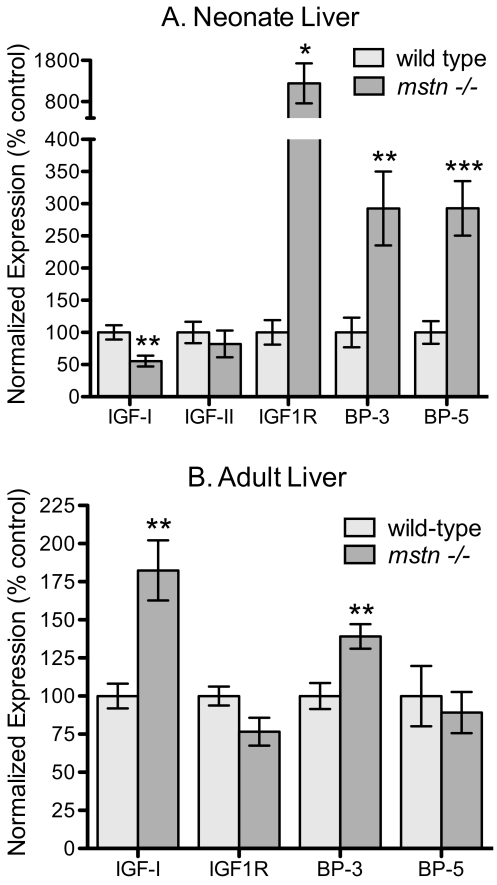

Figure 7. Liver mRNA levels of different IGF/IGFBP axis components in wild type and *mstn-/-* mice. Quantitative RT-PCR comparison was performed as in the legend of Figure 3. (wt n=12, mstn -/- n=12; p=0.05*, 0.01*, 0.001***)

type animals, and these ratios fell to 19%, 34% and 52% in *mstn* knockout animals (TABLE 3).

IGFBP-3 inhibits myoblast proliferation primarily through IGF binding.

Investigation of IGF independent IGFBP-3 effects were conducted with L6 rat myoblasts exposed to increasing doses of IGFBP-3 and mutant analogs, both in the presence and absence of IGF-I. MDGEA IGFBP-3 contains a non-functional mutated metal-binding domain (MBD) necessary for nuclear translocation and known IGFBP-3 specific effects (Firth and Baxter 1999). A 2N+2C IGFBP-3 mutant with four glycine substitutions at residues essential for IGF binding at both the N- and C-terminals of IGFBP-3 was used to gauge the necessity of IGF interactions. Dose-dependent inhibition was observed with both wild type IGFBP-3 and the MDGEA mutant, especially with additional IGF-I stimulation, indicating IGF interactions play an important role in growth regulation (Figure 9). Non-IGF-I binding 2N+2C IGFBP-3 did not significantly alter L6 myoblast proliferation, though dose-dependent inhibition was again observed.

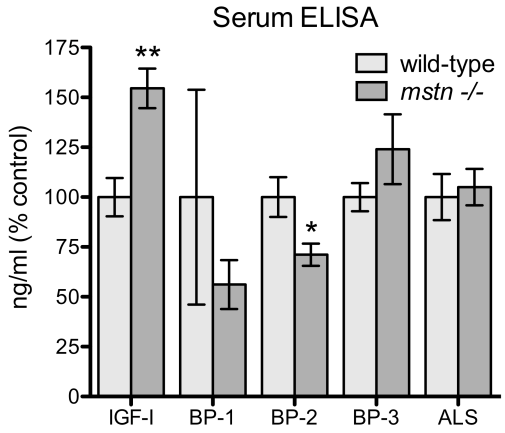

Figure 8. Circulating levels of different IGF/IGFBP axis components in wild type and *mstn -/-* mice. Serum collected from 7 month old wt and *mstn* -/- was analyzed with ELISAs for common IGF axis constituents. Samples were run in duplicate and compared by against % wild type controls. (wt n=8, mstn -/- n=8; p=0.05*, 0.01*, 0.001***)

nM				
	wt	% IGF-I	*mstn*-/-	% IGF-I
IGF-I	27.6±2.6		41.1±2.6	
IGFBP-1	0.2±0.1		0.1±.02	
IGFBP-2	10.8±1.1	39%	7.7±0.6	19%
IGFBP-3	11.3±0.8	42%	14.0±2.0	34%
ALS	20.2±2.3	73%	21.2±1.9	52%
Significant $\alpha \leq .05$				

TABLE 3. Circulating levels of IGF-I, IGFBP-1, IGFBP-2, IGFBP-3 and ALS.

ELISAs were used to quantify IGF axis constituents in circulation from wild type and myostatin null mice. The percentage figures to the right of each value reflect the ratio of that binding protein to IGF-I. (wt n=8, mstn -/- n=8; p=0.05*, 0.01*, 0.001***)

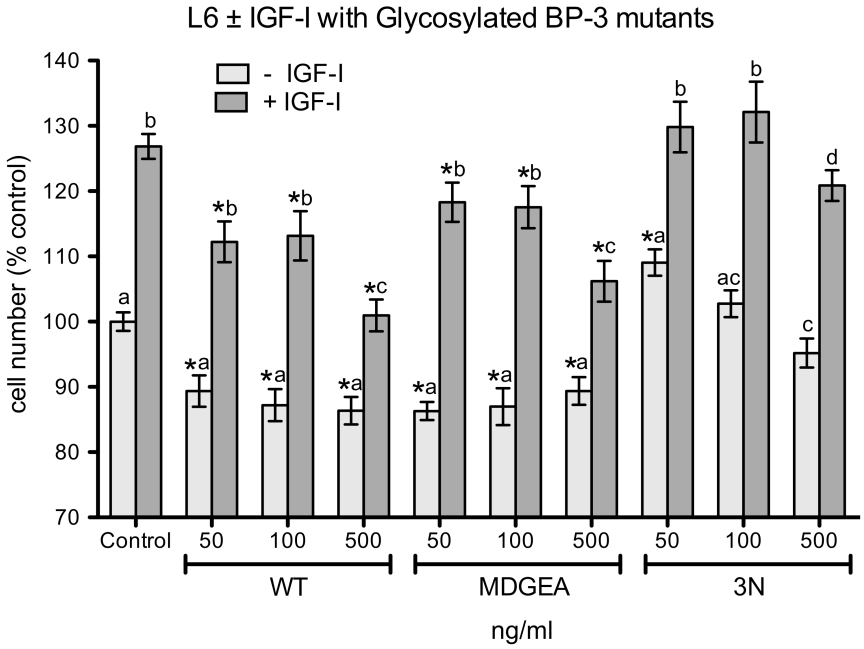

Figure 9. IGFBP-3 inhibition of myoblast proliferation via IGF-dependent and -independent effects. L6 myoblast proliferation was measured in the presence and absence of IGF-I as described in the materials and methods. WT is native glycosylated IGFBP-3, MDGEA is a mutant IGFBP-3 with the inability to nuclear localize, and 3N has three residues necessary for IGF binding substituted with glycine residues, greatly reducing its affinity for IGFs. Samples were run at least twice. Letters indicate differences at α=.05 for each variety of IGFBP-3. (n=16 per value; p=0.05*)

CHAPTER THREE

IMPLICATIONS OF ENDOCRINE MYOSTATIN EFFECTS

While signaling downstream from myostatin has been investigated, interactions with these diverse signaling cascades are by no means fully characterized (Yang, Zhang et al. 2007). Myostatin's action has been long hypothesized to inhibit growth by altering the IGF system, however studies to date have found few connections. Our model offers an explanation for myostatin's role in regulation of organism growth by altering the IGF axis, as well as providing a role in endocrine regulation of growth.

Myostatin reduces IGF sensitivity in neonates

Previous studies have characterized myostatin as a progenitor cell inhibitor, inducing satellite cell differentiation and depleting the pool of cells required for muscle development later in life (Manceau, Gros et al. 2008). Myostatin's role in regulating IGF1R in neonatal skeletal and cardiac tissue indicates that myostatin is responsible for depressing IGF1R action and desensitizing tissues to IGFs. IGF desensitization may be partly responsible for depletion of muscle progenitor pools and may interfere with the dynamic balance between proliferation and terminal differentiation in myoblasts, allowing for quiescent states as Rios *et al.* observed. Additionally, with decreased IGF sensitivity, tissue specific IGF axis components, which include tissue production of IGFBP-3 and -5, are potentially impacted and may alter autocrine/paracrine IGF signaling in these tissues.

Systemic effects may also result from down-regulated IGF1R, as the liver was most impacted by myostatin absence. With myostatin blocking IGF sensitivity,

circulating IGFBP levels will decrease, resulting in clearance of free IGFs and depressing organismal growth.

While IGFBPs were once thought to inhibit IGF action in local environments, work since has demonstrated context sensitive situations where IGFBPs can bolster IGF action by sequestering free IGFs, and after proteolytic cleavage or phosphorylation of IGFBP, release the growth factors close to cell receptors, leading to increased signaling (Ranke and M. 1997; Ma, Pollak et al. 1999; Payet, Wang et al. 2003). Myostatin fundamentally changes expression of both IGF1R and IGFBPs in neonatal tissues.

Myostatin's effect on the IGF axis is temporally regulated

Myostatin expression is high *in utero*, and gradually eases as growth velocity increases, particularly during puberty (Shibata, Matsumoto et al. 2006). According to studies in our lab, the phenotype of neonatal *mstn* -/- mice is indistinguishable from that of wild type animals. However, satellite cell populations affected by myostatin and early impacts on satellite cell number may provide a phenotypic contribution later in life. Alterations in the pattern of IGF expression in adult tissue suggest myostatin regulation of IGF1R drops dramatically, and muscle specific regulation changes as well, with more changes in type-II fibers. Introduction of myostatin takes several hours to down-regulate the Akt/mTOR pathway, indicating intermediate factors, perhaps IGF axis constituents, are required for repression (Yang, Zhang et al. 2007; Amirouche, Durieux et al. 2008).

Myostatin may regulate dimorphic muscle growth through the IGF axis.

Studies have long noted that *mstn* -/- favors growth of glycolytic type II muscle fiber over aerobic type I (Lee 2004; Steelman, Recknor et al. 2006). In gastrocnemius, which is primarily type I muscle fiber, expression of IGF axis components differs

significantly from that of predominantly type II pectoral muscle (Lee 2004). While IGF-I mRNA decreases in the gastrocnemius muscle, expression is so low that this likely has limited bearing on muscle growth, however, IGF1R levels increase significantly in the gastrocnemius, likely increasing type I muscle sensitivity to IGF stimulated growth. However, IGFBP-5 expression is also elevated and may bind and sequester IGFs before they can interact with membrane receptors. The contribution of varying IGF axis constituents may be involved in differing growth between type I and type II muscle, however further study is needed.

Myostatin is an endocrine regulator of the IGF axis.

Circulating myostatin was characterized by Zimmer et al. in 2002, and while many hypotheses abound for its endocrine function, no specific purpose has been attributed to its presence. The liver, which is known to have high ActRIIA and ActRIIB receptor levels, may be affected by circulating myostatin, which provides a straightforward explanation and suggests the potential for a feedback loop from muscles on growth pathways (Woodruff, Krummen et al. 1993; Gold, Zhang et al. 2005).

In *mstn* -/- mice, IGFBP-3 and ALS ratios to IGF drop, leaving more IGF unbound and free to interact with receptors. This likely leads to part of the double muscle phenotype observed in mice, as elevated systemic IGF-I drives muscle growth. Unbound circulating IGF is also proteolyzed without a tertiary complex to protect it, but despite this, levels remain high, suggesting that production is high enough to maintain free IGF despite proteolysis and receptor binding. A model system with a similar elevation in systemic IGF-I was used by Liao *et al.*, who used an inducible IGF-I transgene to increase systemic IGF-I 54% at 8-12 weeks of age. Effects are remarkably

similar to myostatin knockout animals with skeletal muscle hypertrophy and additional effects on pituitary (+54%), heart (+15%), liver (+22%), kidney (+20%) and spleen (+41%) size. Myostatin's role in regulating the IGF axis in knockout animals leads to increased muscle growth, and significantly alters regulation of liver IGF axis constituents. Further work needs to be conducted on other signaling pathways myostatin may regulate in endocrine systems, especially in the liver and other tissues with ActRIB.

Myostatin is a muscle specific feedback mechanism.

Myostatin is a muscular growth inhibitor responsible for dynamically inhibiting muscle growth, thus there is a need to coordinate its action with existing proliferative mechanisms (Lee, Reed et al. 2005; Matsakas, Bozzo et al. 2006). While IGF regulation of myostatin has been discounted in a number of studies, GH alters myostatin transcription, and IGF feedback down regulates GH secretion from the pituitary (Liu, Thomas et al. 2003; Miyake, Hayashi et al. 2007). Therefore, it is plausible that myostatin downregulation of IGFs induces increased GH, which in turn down regulates myostatin, leading to a cyclic feedback on one another's action for the purpose of tying muscle growth and development to organismal growth.

The IGFs are commonly considered important regulators of bone growth as reductions in systemic IGFs dramatically reduce long bone density and length (Yakar, Rosen et al. 2002; Govoni, Baylink et al. 2005). However, while IGFs affect bone growth when dramatically lowered, moderate reductions show almost no effect on bone phenotype (Yakar, Pennisi et al. 2005). Furthermore, circulating IGF has minimal effect on bone growth, as a 54% increase in IGF-I results in only a 2% increase in bone length (Liao, Dearth et al. 2006). However, the same increase in IGF-I stimulates skeletal

muscle hypertrophy by 19% and hyperplasia by 11%, resulting in muscles approximately 33% larger than control animals. Myostatin's feedback on GH through the IGFs may be responsible for muscle specific feedback on organismal growth and tie muscle into systemic regulation of growth.

IGFBP-3 growth inhibition is largely due to IGF binding.

IGFBP-3 levels in cardiac and skeletal muscle decreased with age and likely relieve IGFBP repression of growth as animal's age. The role of IGFBP-3 in muscles is largely to bind and sequester IGFs, limiting IGF1R interaction and growth effects and a decrease will allow heightened growth response. Other studies have characterized IGFBP-3 as an IGF-independent growth inhibitor in a number of cell lines, and some studies to date have implicated other TGF-β members in regulation IGFBP-3 levels (Kansra, Ewton et al. 2000; Pampusch, Kamanga-Sollo et al. 2003; Kamanga-Sollo, Pampusch et al. 2005). While myostatin knockout effected expression, when comparing gastrocnemius and pectoral muscle no obvious conclusions can be drawn on the effects of IGFBPs on proliferation. *In vitro* experiments with L6 myoblasts demonstrated primarily IGF-dependent growth inhibition, and suggest that in proliferating myocytes the majority of IGFBP-3's action is accomplished through direct binding to IGFs. As a function of signaling, myostatin may have effects on autocrine/paracrine IGF signaling, as well as effects modifying IGFs from circulation as a result of altered IGFBP-3 and IGFBP-5 levels, however further study is needed.

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