

MYOSTATIN NEGATIVELY REGULATES CARDIAC MUSCLE GROWTH,  
DEVELOPMENT AND PERFORMANCE

By

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To the Faculty of Washington State University:

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Abstract

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Myostatin is a known negative regulator of skeletal muscle growth and is suspected to similarly influence cardiac muscle. To test this hypothesis, we determined the effect of myostatin on the proliferation and differentiation of rat H9C2 cardiomyoblasts. Myostatin inhibited basal and insulin-like growth factor (IGF)-I stimulated proliferation in a dose dependent manner and partially blocked retinoic acid-induced differentiation. These cells expressed a full complement of myostatin receptors (Acvr2, Acvr2b, ALK4 & ALK5) and binding proteins (follistatin & follistatin-like protein (FSTL)-3), although some of the expression patterns differed considerably from those in skeletal muscle cells. To investigate myostatin's effect on cardiac function, echocardiography was performed on myostatin null and wild-type mice. Measurements of left ventricle internal diameter, but not wall thickness, were larger in null mice, which resulted in greater end diastolic and systolic volumes. Such differences are indicative of eccentric hypertrophy. Isoproterenol stress tests revealed a greatly enhanced response in null mice, whose hearts were also heavier. Fetal gene expression was similarly low in both wild-type and null mice further indicating that the eccentric hypertrophy was

physiological and not pathological. Expression of follistatin and FSTL-3 were elevated in infarcted cardiac muscle of wild-type mice, although only follistatin levels were significantly different from non-infarcted muscle. These studies suggest that myostatin is a negative regulator of cardiac muscle growth and that the myostatin null mouse is a good model for investigating mechanisms of physiological hypertrophy. They further suggest that limiting myostatin action or bioavailability could improve cardiac muscle repair and performance.

Additionally, myostatin control over insulin like growth factors (IGF) and IGF binding protein (IGFBP) levels was investigated. In neonatal mice, IGF-II, and IGF-IR were significantly elevated by approximately 70% and 250%, respectively, whereas in adult mice IGF-IR was decreased by 40%. Changes in IGF/IGFBP production could presumably affect other tissues as well. Thus, we investigated the effect of myostatin knockout on body size over a period of seven months by measuring tibia epiphyseal plate width, tibia length, and tail length. However, there was no significant difference in these parameters between myostatin null and wild type mice at any time period.

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## CHAPTER ONE

### INTRODUCTION

#### ***Discovery of myostatin and its null phenotypes***

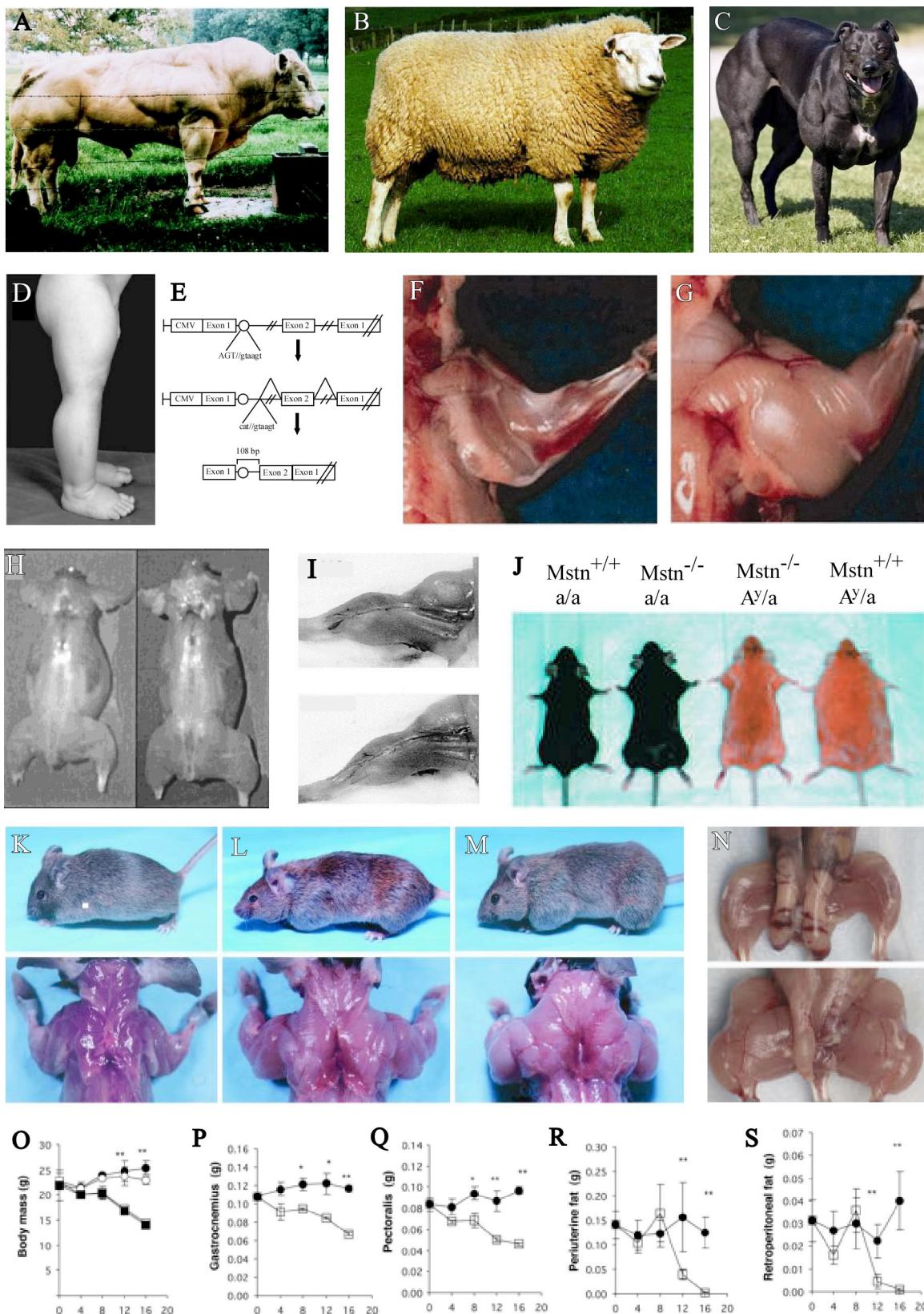
Chalones are chemical messengers (i.e. hormones, growth factors, cytokines, etc) that help control and optimize the size of specific target tissues by inhibiting cellular growth.

William S. Bullough coined the term and predicted the existence of chalones in the 1960's (Bullough, 1962). There was no definitive evidence that such hormones existed until 1997 when myostatin was discovered during a search for novel members of the TGF- $\beta$  superfamily of growth and differentiation factors (McPherron et al., 1997). Myostatin expression is limited to skeletal muscle, and targeted disruption of the gene (a.k.a. "knockout") through homologous targeting in mice, removing the entire myostatin coding frame and replacing it with a *neo* cassette, resulted in an approximately 200% increase in muscle mass (McPherron et al., 1997) (Fig. 1). This is due to both larger muscle fiber (hypertrophy) and increased fiber number (hyperplasia), and mice exhibited increased limb strength as well (Bogdanovich et al., 2002; McPherron et al., 1997; Wagner et al., 2002; Whittemore et al., 2003) Researchers concluded that myostatin is a skeletal muscle chalone.

The myostatin null phenotype was first described in cattle, although its genetic basis was only revealed with the discovery of myostatin. The Belgian Blue, Piedmontese, and Marchigiana breeds are described as "double muscled," although the actual muscle increase is only about 20% greater than normal muscled animals (Fig. 1) (Grobet et al., 1997). Recent genetic studies have

proven that all three breeds possess mutations in the coding region of myostatin that results in mutated and inactive proteins. The Belgian Blue has an 11-bp deletion, a frameshift mutation that results in a premature stop codon and a shortened protein lacking its bioactive domain (Grobet et al., 1997; Kambadur et al., 1997; McPherron and Lee, 1997). The Piedmontese has a G-A transition that changes a cysteine at amino acid 314 into a tyrosine, mutating an important secondary structure and rendering the entire myostatin protein inactive (Kambadur et al., 1997; McPherron and Lee, 1997). The Marchigiana myostatin coding region also has a G-A transition, however, in this breed the result is a premature stop codon (Marchitelli et al., 2003)

Myostatin null phenotypes have also been described in sheep, dogs and in a young boy (Fig. 1). The Texel sheep is documented as being double muscled and results from a G-A transition in the 3'-UTR (Clop et al., 2006; Johnson et al., 2005). This mutation, instead of inducing premature stop codons or improper protein folding, creates a site for microRNAs to bind and initiate degradation of the myostatin transcript (Clop et al., 2006). Another double muscled animal is the bully whippet, long believed to be a deleterious mutant of the sleek racing dog. A recent study discovered that bully whippets have a 2 bp deletion that results in a premature stop codon at amino acid 313 that produces a biologically inactive protein (Mosher et al., 2007; Shelton and Engvall, 2007). Homozygotes for the mutation have the extreme muscle hypertrophy that led to many dogs being euthanized as “mutants”, however, the trait had been selected for over generations by breeders as the heterozygotes have only mild increases in muscle definition, perform better as racing dogs and are preferred for showing (Mosher et al., 2007). The myostatin null phenotype has also been observed in a human. In 2000, a German boy was born with obvious muscle hypertrophy. Tests showed no disease pathology and a



**Figure 1. Myostatin Phenotypes** (A) Muscle hypertrophy in Belgian Blue cattle breeds is due to an 11-nucleotide deletion within the third exon of the bovine myostatin gene (McPherron and Lee, 1997). (B) In the Texel sheep it is due to a nucleotide transition that creates a microRNA binding site in the 3'-UTR. (C) The “bully whippet” is a result of a homozygous two base pair deletion that results in a premature stop codon. (D & E) Leg musculature of a 7-month old infant boy with a nullifying splice site mutation in the myostatin gene (E) (Schuelke et al., 2004). (F & G) Forearm musculature of a wild type (F) and myostatin “knock-out” (G) mouse (McPherron et al., 1997). (H) Musculature of a wild type mouse (left) and transgenic mouse overexpressing LAP (right) (Yang et al., 2001). (I) Muscle hypertrophy due to a dominant negative form of myostatin (upper panel) compared to the musculature of a wild type mouse (lower panel) (Zhu et al., 2000). (J) Effect of myostatin genotype on fat deposition in Agouti lethal yellow obese mouse (McPherron and Lee, 2002). (K-M) Musculature of wild type (K) mice, transgenic mice overexpressing a dominant negative activin receptor alone (L), or in addition to follistatin (M) (Lee and McPherron, 2001). (N) Hind leg musculature of wild type (upper panel) and a myostatin knock-out/follistatin transgenic mouse (Lee, 2007). (O-S) Cachexic effect of overexpressing LAP on body weight and skeletal muscle weights over time (days), O = open circles, CHO-controls; solid squares, CHO-myostatin; P-S = solid circles, CHO-control; Open squares, CHO-myostatin (Zimmers et al., 2002).

genetic study revealed a splice site mutation in exon 1 that results in a severely truncated protein that lacks a functional bioactive domain (Rodgers and Garikipati, 2008; Schuelke et al., 2004).

Model organisms overexpressing myostatin have the opposite phenotype of the myostatin null models (Fig.1). Mice subcutaneously injected with Chinese Hamster Ovary (CHO) cells overexpressing myostatin became extremely gaunt, losing about 33% of their body mass, which was due to decreased skeletal muscle fiber diameter (Zimmers et al., 2002), a condition known as cachexia, which is often observed in terminally ill patients. Transgenic mice with skeletal muscle-specific overexpression of myostatin also have significantly reduced muscle mass, losing approximately 20% of skeletal and cardiac muscle mass due to decreased fiber diameter and number (Reisz-Porszasz et al., 2003).

### ***Structure, functional relationships and receptor signaling***

All the mutations occurred in domains critical to myostatin function. These domains, conserved amongst the TGF- $\beta$  superfamily, include an RSRR sequence which signals the cleavage of the N-terminal from the C-terminal, and nine cysteine residues that form a cysteine knot (Lee, 2004; Lee and McPherron, 2001; Thies et al., 2001). Myostatin is also 90% similar to another TGF- $\beta$  family member, GDF-11, that is processed in a pattern similar to myostatin (Gamer et al., 1999; Nakashima et al., 1999). The cleaved C-terminal fragment, also known as the latent associated peptide (LAP), stays in a non-covalent linkage with the myostatin N-terminal dimer. This complex is referred to as the latent complex and is inactive and unable to bind to its receptor until it is proteolytically cleaved, most likely by a member of the toll-like

family of metalloproteinases (Lee and McPherron, 2001; Thies et al., 2001; Wolfman et al., 2003).

TGF- $\beta$  ligands signal through serine/threonine kinase receptors, which phosphorylate Smad proteins directly. Myostatin specifically signals through the activin type II receptors (ActRIIA and ActRIIB) which recruit activin type I receptors (ALK-4 and ALK-5) and phosphorylate Smad-2 and Smad-3 (Langley et al., 2002; Lee and McPherron, 2001; Rebbapragada et al., 2003; Thies et al., 2001). These receptor Smads form a complex with Smad-4, a co-Smad, which translocates to the nucleus (Massague, 1998; Zhu et al., 2004). The Smad 2/3/4 complex can activate or inhibit transcription factors that control differentiation and proliferation of myoblasts (Langley et al., 2002; McKoy et al., 2007). Specifically, p21 is upregulated, Cdk2 is downregulated, and Rb is dephosphorylated (Rios et al., 2001; Taylor et al., 2001; Thomas et al., 2000). Differentiation is blocked in C2C12 myoblasts by downregulating MyoD and Myf-5, which normally stimulate the expression of myogenin and other transcription factors that induce differentiation of myoblasts (Joulia et al., 2003; Langley et al., 2002; Rios et al., 2002). There is evidence, however, that rather than halting differentiation *per se*, myostatin induces cell cycle withdrawal and cellular quiescence (McCroskery et al., 2003; Wagner et al., 2005). These effects are not limited to the C2C12 model, as similar studies with primary bovine fetal myoblasts (Thomas et al., 2000) suggest that myostatin's actions are conserved at least among mammals (Rodgers and Garikipati, 2008). Additionally, protein synthesis has been observed to be inhibited by myostatin (Taylor et al., 2001).

*In vivo*, the target of these actions are likely muscle satellite cells, the quiescent stem cell population (Lee, 2004; McCroskery et al., 2003). Downregulation of the proliferation and

differentiation of satellite cells would certainly limit the growth and development of wild type muscle. Muscle fiber analyses of myostatin knockout mice show a higher number of satellite cells per unit length and a higher proportion of activated satellite cells (McCroskery et al., 2003).

### ***Inhibitors of myostatin action***

Due to myostatin's potent action, bioavailability is tightly controlled in the cell by a series of cleavage events (see above). Myostatin's inactivity until cleavage of the propeptide indicates the LAP is a major inhibitor of myostatin. *In vitro* and *in vivo* studies have reported the effects of the LAP on myostatin, most dramatically in transgenic mice overexpressing the LAP, which display the double muscled phenotype of a myostatin knockout mouse (Fig. 1) (Qiao et al., 2008). There are additional myostatin inhibitors including follistatin, follistatin like (FSTL)-3, and growth/differentiation factor associated serum protein (GASP)-1. Follistatin, known to bind to and inactivate multiple members of the TGF- $\beta$  superfamily, including GDF-11, activin, and BMP-4 (de Winter et al., 1996; Fainsod et al., 1997; Iemura et al., 1998; McPherron et al., 1997; Nakamura et al., 1990; Yamashita et al., 1995), is clearly capable of inhibiting myostatin action as well (Fig. 1) (Lee and McPherron, 2001; Zimmers et al., 2002). Transgenic overexpression of follistatin results in double muscling reminiscent of myostatin knockout phenotypes (Lee and McPherron, 2001), and follistatin knockout mice show reduced muscle mass (Matzuk et al., 1995). Additionally, simultaneous overexpression of follistatin and myostatin knockout results in “quadruple muscled” mice, highlighting follistatin’s control over other growth inhibitors besides myostatin (Lee, 2007).

FSTL-3 is another TGF- $\beta$  superfamily inhibitor, known to bind to and inactivate the signaling actions of activin, BMP-2 and myostatin (Hayette et al., 1998; Hill et al., 2002; Tsuchida et al., 2000). Overexpression of FSTL-3 also results in a double-muscled phenotype in transgenic mice (Lee, 2007). The third myostatin inhibitor, GASP-1, was discovered bound to myostatin and BMP-11 through affinity purification and mass spectrometry (Hill et al., 2003). It contains a follistatin domain and multiple protease inhibitor domains, which could interfere with cleavage of the LAP or processing myostatin's propeptide (Hill et al., 2003). As GASP-1 was found bound to both mature myostatin and the propeptide, it is possible GASP-1 binds a myostatin precursor and never releases, or that its follistatin domain lends it multiple functions (Hill et al., 2003); the full role of GASP-1 is not yet fully elucidated.

#### ***Tissue-specific expression and potential non-muscle actions***

Myostatin is found almost exclusively in mammalian skeletal muscle (McPherron et al., 1997). Myostatin was detected by whole mount *in situ* hybridization in embryonic mouse somites at 9.5 days post-coitum (p.c) and by 10.5 days p.c. myostatin appeared to be restricted to the myotome compartment (McPherron et al., 1997). Northern analysis of adult mouse tissues indicate that myostatin is expressed in all skeletal muscle, although at varying levels (McPherron et al., 1997).

By contrast, myostatin is widely expressed in most tissues of bony fish (Amali et al., 2004; Garikipati et al., 2006; Kerr et al., 2005; Maccatrazzo et al., 2001; Ostbye et al., 2001; Radaelli et al., 2003; Rescan et al., 2001; Roberts and Goetz, 2001; Rodgers and Weber, 2001; Rodgers et al., 2001). It is also highly expressed in different chicken tissues including the liver,

heart brain and intestine (Kubota et al., 2007; Sundaresan et al., 2007). No tissue specific function has been found for myostatin in these non-mammalian vertebrates (Kerr et al., 2005; Kubota et al., 2007; Maccatrazzo et al., 2001; Ostbye et al., 2001; Rescan et al., 2001; Roberts and Goetz, 2003; Sundaresan et al., 2007) although cultures with chick limb buds and embryos indicate that myostatin does inhibit proliferation and differentiation (Amthor et al., 2002; Manceau et al., 2008). This suggests that myostatin has general developmental effects, and its tissue-specific role has evolved more recently with mammals.

Its ubiquitous expression in fish and birds suggests that myostatin may have non-skeletal muscle actions in mammals, as well. Myostatin is detectable by sensitive RT-PCR in cardiac muscle and was most highly expressed in the Purkinje fibers (Sharma et al., 1999). RNA *in situ* hybridization has shown that myostatin is also expressed in the valve leaflets of embryonic mouse hearts, complementary to the myostatin binding proteins follistatin and follistatin-like gene (Takehara-Kasamatsu et al., 2007). Circumstantially, these studies suggest a role for myostatin in regulating cardiac muscle as well as skeletal muscle.

### ***Preliminary evidence for myostatin function in cardiac muscle***

Recent studies with cardiomyoblasts indicate that myostatin inhibits proliferation, as it does with skeletal muscle (Artaza et al., 2007; McKoy et al., 2007), studies that have been replicated in our own lab (see Chapter 2). Cultures of developing embryonic rat cardiomyoblasts, which still proliferate, express significantly lower concentrations of myostatin than non-proliferative cells from neonates (McKoy et al., 2007). In fact, myostatin expression is inversely correlated with age and proliferation capacity in these cells is six times higher in

embryonic heart cells than in cells from 10 day old mice. These studies suggest that myostatin may also regulate the development of cardiac muscle.

Myostatin's receptors and associated inhibitors are also expressed in cardiac muscle. Artaza *et al* detected ActRIIB with real-time PCR in cell cultures of rat cardiomyocytes (Artaza et al., 2007). Follistatin has been detected in developing chicken hearts using *in situ* hybridization (van den Berg et al., 2007) and in developing mouse hearts (Takehara-Kasamatsu et al., 2007). FSTL-3 is also expressed throughout the heart (Takehara-Kasamatsu et al., 2007; Tsuchida et al., 2000; Tsuchida et al., 2004). Preliminary studies in our lab also identified follistatin, follistatin like-3, Acvr2, Acvr2b, ALK4, ALK5, and BAMBI in rodent cardiac muscle (see Chapter 2). However, *in vivo* studies report conflicting effects of myostatin on cardiac tissue. One study found decreased whole heart weight in transgenic mice overexpressing myostatin, suggesting that the increase in myostatin was negatively regulating the development of cardiac tissue (Reisz-Porszasz et al., 2003). Another study found decreased size in transgenic mice with heart-specific overexpression of myostatin, and increased size in myostatin knockout mice, when compared to wild-type mice (Artaza et al., 2007). Yet another study found no difference in ventricular mass, heart to body weight ratio, or cardiomyocyte area between myostatin knockout and wild type mice (Cohn et al., 2007). Left ventricular ejection fraction did not differ between transgenics overexpressing or lacking myostatin, or wild type mice, an indication that cardiac function was not affected by changes in myostatin expression (Artaza et al., 2007; Cohn et al., 2007).

The fact remains that myostatin is expressed in the heart and continues to be a point of interest for treating heart injuries like congestive heart failure or myocardial infarction.

Cardiomyocyte proliferation and differentiation ceases postnatally. Thus, any trauma to the heart is particularly deleterious because the tissue cannot recover. Western blot analysis and real-time PCR show that myostatin expression in the left ventricle of rats does increase after an aorto-caval shunt was created to mimic volume-overload heart failure (Shyu et al., 2006). Furthermore, the drugs N-acetylcysteine, doxazosin, and carvedilol not only ease the effects of heart failure, but decrease myostatin expression as well (Shyu et al., 2006). In another study, Western Blot analysis and immunostaining showed that myostatin expression increased in sheep hearts that had myocardial infarction induced, particularly in peri-infarcted areas (Sharma et al., 1999). Therefore, inhibition of myostatin to encourage cardiac tissue regrowth and recovery has serious clinical implications.

Exercise is another time period in which muscle mass increases, including heart muscle. In rodents, swimming, wheel running, and use of a treadmill have all been shown to decrease myostatin mRNA in skeletal muscle (Matsakas et al., 2006). One study also analyzed cardiac myostatin mRNA levels after rats underwent four weeks of swim training, although myostatin levels actually increased (Matsakas et al., 2006). Matsakas et al found this curious, but thought it might be similar to findings that myostatin is higher in injured hearts, or may be a chalone of insulin-like growth factor (IGF)-I in the heart. This conclusion was also reached by Shyu et al when myostatin RNA levels increased after subjecting rat cardiomyocytes either to IGF-I treatment or to cyclic stretch (Shyu 2005).

### ***Physiological vs. pathological hypertrophy of the heart***

Cardiac hypertrophy is an enlargement of the heart muscle, through cellular hypertrophy and occasionally, fibrosis (McMullen and Jennings, 2007; Catalucci et al., 2008). The initial development of the heart is accomplished through cardiomyoblast proliferation, but shortly after birth further increases in heart size are accomplished solely through hypertrophy of the existing cells. Cardiac muscle does not contain significant numbers of stem cells or satellite cells, as skeletal muscle does, so hypertrophy is necessary for growth. The term “cardiac hypertrophy”, however, is usually associated with pathological conditions and poor prognosis. By contrast, aerobic exercise can also cause cardiac hypertrophy, termed physiological hypertrophy, and has distinct hallmarks that distinguish it from pathological hypertrophy, which often occurs after infarction or volume overload failure. These two general types of hypertrophy differ significantly and are labeled pathological and physiological hypertrophy (Table 1).

Pathological hypertrophy is associated with fibrosis, which further reduces the functionality of the heart tissue (Catalucci et al., 2008; Frey and Olson, 2003; McMullen and Jennings, 2007). Heart walls thicken, reducing the volume of the heart chambers; concentric hypertrophy is a hallmark of such pathological hypertrophy (Grossman et al., 1975; McMullen and Jennings, 2007). Additionally, there is an upregulation of fetal genes involved with heart development, atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), skeletal  $\alpha$ -actin, and  $\beta$ -myosin heavy chain (MHC) (Catalucci et al., 2008; McMullen and Jennings, 2007; Molkentin and Dorn, 2001). Initially, these changes compensate for injured tissue and heart function is maintained, but after a short time, function is significantly reduced; contraction and relaxation decrease and ejection fraction drops (Catalucci et al., 2008; Levy et al., 1990; McMullen and Jennings, 2007; Zak, 1983).

As stated above, not all hypertrophy is pathological and life-threatening. Athletes also display cardiac hypertrophy (McMullen and Jennings, 2007; Catalucci et al., 2008; Richey and Brown, 1998; Schaible et al., 1986; Wisloff et al., 2002; Pluim et al., 2000; Zak, 1983). Athletes involved with aerobic exercise display eccentric hypertrophy, with a proportional increase in chamber size and wall thickness; whereas athletes involved with anaerobic exercise, such as weight lifting, display concentric hypertrophy (McMullen and Jennings, 2007; Catalucci et al., 2008; Grossman et al., 1975; Pluim et al., 2000; Zak, 1983). In contrast to pathological hypertrophy, there is a distinct lack of cardiac fibrosis, nor is there an upregulation in ANP, BNP, skeletal  $\alpha$ -actin or  $\beta$  MHC (McMullen and Jennings, 2007). Ejection fraction remains the same or is increased, and improved contraction allows for more oxygen consumption (Catalucci et al., 2008; Christensen, 1983; Raskoff et al., 1976). This physiological hypertrophy is actually desirable, and is associated with improved cardiac function.

Myostatin's role in either hypertrophy is still under consideration. Myostatin increases after infarction or volume overload heart failure (Sharma et al., 1999; Shyu et al., 2006), though studies disagree on whether it increases or decreases after exercise training in rodents (Matsakas et al., 2006; Shyu et al., 2005). Our preliminary studies suggest that the hearts of myostatin knockout mice have eccentric hypertrophy and improved function, which not only implies that myostatin can control heart morphology, but myostatin-blocking technologies could have serious clinical or performance applications.

### ***Endocrine control of cardiac hypertrophy***

Just as there are two generally different types of cardiac hypertrophy, each arises from two different signaling pathways. Pathological hypertrophy is regulated by signaling through G-

protein coupled receptors (GPCR) including those for endothelin (ET)-1, angiotensin (Ang)-II or catecholamines (Catalucci et al., 2008; McMullen and Jennings, 2007). The catecholamines, epinephrine and norepinephrine, are hormones released by the adrenal glands when an organism is under stress and causes heart rate acceleration, nervous system stimulation, and other physiological stress responses (Gardner, 2007). Physiological hypertrophy is accomplished through the near-ubiquitous Akt molecule which is activated via IGF-I and phosphoinositide-3 kinase (PI3K) signaling, a system involved in organismal and cellular growth (Catalucci et al., 2008; Walsh, 2006).

Catecholamines, ET-1 and Ang-II have been observed to increase during pathological hypertrophy (Catalucci et al., 2008; McMullen and Jennings, 2007). All three are known to activate various GPCRs, which are bound to  $G_{\alpha}$  proteins (Catalucci et al., 2008; McMullen and Jennings, 2007). Depending on which  $G_{\alpha}$  subtype is activated either pathological or physiological hypertrophy can result. For example, catecholamine signaling through  $\beta_1$ -adrenergic receptors (AR) are coupled to  $G_s$  proteins, which leads to pathological hypertrophy (Catalucci et al., 2008; McMullen and Jennings, 2007). However, the  $\beta_2$ -AR is coupled to either  $G_s$  or  $G_i$ , and  $G_i$  activates PI3K and Akt, which leads to physiological hypertrophy (Catalucci et al., 2008; McMullen and Jennings, 2007). In this way, catecholamines can be linked to increased cardiac function in athletes or to heart failure after a stress event like myocardial infarction or hypertension.

Endocrine IGF-I is produced mostly by the liver in response to growth hormone and to a lesser extent insulin and binds tyrosine kinase receptors, which activate PI3K

**Table 1. Characteristics of Pathological and Physiological Cardiac Hypertrophy.**

	Pathological		Physiological	
	Eccentric	Concentric	Eccentric	Concentric
<b>Morphology</b>	Thickened heart walls, proportional chamber enlargement	Thickened heart walls	Thickened heart walls, proportional chamber enlargement	Thickened heart walls
<b>Cause</b>	Volume overload due to hypertension	Pressure overload, myocardial infarction, valve disease	Volume overload due to aerobic exercise, post-natal growth, pregnancy	Pressure overload due to anaerobic exercise
<b>Histology</b>	Increased fibrosis and cell size		Increased cell size	
<b>Fetal Gene Expression</b>	Increase in ANP, BNP, and $\beta$ -MHC; decrease in $\alpha$ -MHC and SERCA		Stable	
<b>Function</b>	Decrease in Stroke vol, EF, FS, Diast vol, Syst vol, Ao velo, Ao acel*		Increase in Stroke vol, EF, FS, Diast vol, Syst vol, Ao velo, Ao acel*	

\* Stroke vol – stroke volume; EF – ejection fraction; FS – fractional shortening; Diast vol – diastolic volume, Syst vol – systolic volume; Ao velo – aortic velocity; Ao acel – aortic acceleration

(Catalucci et al., 2008; McMullen and Jennings, 2007). In turn, PI3K phosphorylates phosphoinositol phosphates (PIP) which bind and ultimately activate Akt, and its downstream effectors, the ribosomal S6 kinases (Catalucci et al., 2008; McMullen and Jennings, 2007). IGF-I knockout or IGF-I receptor knockout mice are significantly smaller than wild-type counterparts (Walsh, 2006). Therefore, it should come as no surprise that activated Akt is detected after exercise training in mice (Kemi et al., 2004), that IGF-I is elevated in athletes with physiological hypertrophy (Neri Serneri et al., 2001) and that dominant-negative PI3K will abolish physiological hypertrophy from exercise (McMullen et al., 2003).

Understanding the signaling pathways involved with cardiac hypertrophy can help determine the pathological or physiological effect of myostatin. Preliminary studies from our lab suggest that myostatin knockout mice are more sensitive to the synthetic equivalent of epinephrine, isoproterenol, and show increased heart rate and cardiac output in accordance with physiological hypertrophy (see Chapter 2). It is unclear whether this sensitivity is due to an increased number of  $\beta$ -AR, heightened signaling pathways, or the binding kinetics of isoproterenol with its receptors. Nevertheless, myostatin's role clearly appears to enhance beneficial growth processes in mice and may lead to future therapies for treating myocardial infarction, heart failure associated with muscular dystrophy, or other chronic heart pathologies.

## **CHAPTER TWO**

### **MYOSTATIN NEGATIVELY REGULATES CARDIAC MUSCLE GROWTH, DEVELOPMENT, AND PERFORMANCE**

#### ***Introduction***

Myostatin has been a proven regulator of skeletal muscle proliferation and differentiation, and has recently been determined to be present in cardiac muscle, along with its binding proteins (Artaza et al., 2007; Sharma et al., 1999; Takehara-Kasamatsu et al., 2007; Tsuchida et al., 2007). This suggests that myostatin may additionally influence the growth and development of cardiac muscle. The cytokine is differentially expressed during development (McKoy et al., 2007) and expression has been shown to increase following injuries to the heart, such as myocardial infarction or volume-overload heart failure (Modesti et al., 2004; Sharma et al., 1999; Shyu et al., 2006). These changes are similar to those occurring in damaged skeletal muscle (McCroskery et al., 2003; Sharma et al., 2001) and suggest that myostatin may also influence cardiac muscle repair. Indeed, recent studies with Akt transgenic mice (Cook et al., 2002), *in vitro* models of cyclic stretch and IGF-I stimulated cardiomyocytes (Shyu et al., 2005) all suggest that myostatin not only regulates some cardiac muscle growth processes, but that it may function as a cardiac chalone as it does in skeletal muscle (Gaussian and Depre, 2005). Myostatin expression is elevated in all of these models and may therefore provide a negative feedback mechanism to limit cardiac muscle growth and thus, pathological hypertrophy. A better understanding of myostatin's functional role in regulating cardiac muscle growth and development could therefore help in developing novel therapies for treating cardiac disorders.

Reported herein is the functional assessment of cardiac performance in myostatin null mice. Echocardiography indicates that stress-induced cardiac performance is enhanced in myostatin null mice, which also have enlarged hearts. The myostatin null mouse, therefore, appears to be a novel and unexplored model of physiological, but not pathological, cardiac hypertrophy.

### ***Materials and Methods***

*Cell culture.* H9C2 cardiomyoblasts, a clonal line derived from embryonic rat heart, were cultured at 37 °C in a 5% CO<sub>2</sub> humidified atmosphere in Dulbecco's modified Eagle's medium (DMEM) (Sigma, [www.sigmaldrich.com](http://www.sigmaldrich.com)) supplemented with 10% fetal bovine serum (FBS), 0.2 mM glutamine, 100 IU/ml penicillin, 0.1 mg/ml streptomycin and 250 ng/ml amphotericin B. Proliferation assays were performed on cells originally plated at 50% confluence and in serum-free DMEM supplemented with 2.7, 11 or 55 nM long R3 (LR3) IGF-I (Diagnostic systems laboratory, [www.dslabs.com](http://www.dslabs.com)) for 24 and 48 h. Cells were also cultured with 2.7 nM LR3 with 0, 2.7 nM or 11 nM human myostatin (Metamorphix, [www.metamorphixinc.com](http://www.metamorphixinc.com)) for 48 h of incubation. At the end of the incubation period, total cell number was measured using the Cell Titer 96 proliferation assay (Promega, [www.promega.com](http://www.promega.com)). To differentiate, cells were grown to 70% confluence in DMEM supplemented with 1% FBS and stimulated with 10 nM retinoic acid (Sigma) daily as described (Menard et al., 1999). Briefly, retinoic acid was diluted in the dark with DMSO, aliquoted and stored as 1 mM stock solutions and used at a final concentration of 10 nM. To determine the effect of myostatin on H9C2 differentiation, cells were cultured in differentiation medium for 3 to 6 days with or without addition of 11 nM myostatin. Myostatin was added at the beginning of the experiment and

additionally on day 3 in the 6-day group. Quantitative RT-PCR was performed to measure the expression of the differentiation marker equilibrative nucleoside transporter (ENT)-1.

*Animals.* Rat tissue samples were obtained from animals bred at the experimental animal laboratory building, Washington State University. Myostatin null mice were generously donated by Prof. Se-Jin Lee, Johns Hopkins University, and were maintained in the same facility. Mice used in the infarction studies were similarly housed in facilities at the University of Washington. All animals were housed in environmentally controlled rooms with 12 h daily light and their use was performed according to protocols pre-approved by the Institutional Animal Care and Use Committees (IACUC) at these universities.

Myocardial infarctions were induced in wild-type mice using the ischemia-reperfusion model as previously described (Gaussin and Depre, 2005). Briefly, mice were anesthetized by injecting a cocktail of 60 mg/g ketamine, 50 mg/g pentobarbital and 0.2 U/g heparin and prepared for surgery. Ophthalmic ointment was applied and mice were placed on a 38°C water blanket, intubated and attached to a ventilator. A left thoracotomy was performed through the fifth intercostal space, the chest was exposed with a retractor and the pericardium was incised. Ischemia was achieved by tying a suture around a sterile polyethylene glycol tubing that was placed on top of the proximal left anterior descending artery for 30-60 minutes. Successful ischemia distal to the ligation site was verified visually while reperfusion was achieved by removing the tubing and suture.

*Qualitative & quantitative RT-PCR.* Total RNA was extracted from cells, cardiac muscle and skeletal muscle using Trizol reagent (Invitrogen, [www.invitrogen.com](http://www.invitrogen.com)). Tissues were first

snap frozen in liquid nitrogen, pulverized while frozen and homogenized in Trizol. RNA was treated with DNase (DNase RQ-1, Promega) and its integrity was assessed by agarose gel electrophoresis. Reverse transcription was performed on 1 µg RNA from infarcted mouse tissue and 5 µg RNA from rat tissue and cells using the First Strand cDNA synthesis kit (Invitrogen). In all assays, template loading was controlled by adding an equal volume of cDNA to a master mix for each sample, which was then aliquoted before the addition of gene-specific primers.

Primers and the annealing temperatures used in the qualitative RT-PCR assays for Acvr2, Acvr2b, ALK-4, ALK-5, follistatin, FSTL-3, GASP-1, equilibrative nucleoside transporter (ENT)-1, bone morphogenic protein/activin membrane-bound inhibitor (BAMBI) and β-actin are included in Table 2. These assays were performed with RNA from adult rat heart and skeletal muscle and from proliferating and differentiating H9C2 and L6 myoblasts. The PCR protocol included an initial denaturation of 94 °C for 1 min, followed by 30, 35 or 40 cycles of 94 °C for 30 s, a gene-specific annealing temperature for 30 s and 72 °C for 90 s. Relative, but not absolute, differences in expression levels were reliably estimated as no differences in the expression of the loading control, β-actin, was observed.

Quantitative RT-PCR assays were conducted using the iCycler iQ Real-time PCR Detection System (Bio-Rad, [www.biorad.com](http://www.biorad.com)) and gene specific primers for ENT-1 and elongation factor (EF)-2 (Table 2). To determine myostatin's effect on H9C2 cell differentiation, cDNA was amplified at 95 °C for 10 s, followed by 57 °C for 30 s and 72 °C for 30 s using primer concentrations that were empirically determined (ENT-1 FP = 400 nM, ENT-1 RP = 200 nM; EF-2 FP = 200 nM, RP = 200 nM). Melt curve analysis was performed with each

**Table 2. Primer sequences and annealing temperatures**

Gene Product	Forward / Reverse Primer Sequences (5' - 3')	Temp. (°C)
Acvr2a	TGGCGITCGCCGTCTTCTTATC / AGTGTGGACAGATGGTGCAAC	60
Acvr2b	CAGGGTGGCACCAAGACGGTAC / TCGATGGTCACGCAGAGCTGG	55
ALK-4	TGCCCAAGTGGACACCTCAAGG / TGGACTCCTCCAGAATTGCACCTC	60
ALK-5	CCGAGACAGGCCATTGTTGT / TCGTGGATCCGCCAATGGAAC	60
FS	GCGGGCTCTGCCTCTGCTG / TTCCACCACCGCACTGGATGTC	70
FSTL-3	TCTGCTGGTACTTGGGGCCAC / AGCCTGGTCCAACTTCACCCA	65
GASP-1	GCTCCCCTTCGAGGCCCTAGC / GTCCTCACTGTCTGGGGGCTTCAG	63
MSTN	GCTGATTGCTGCTGCCAGTGG / GAGCACCCACAGCGGTCTACTACC	60
β-actin	CGCTGCGCTGGTCGTCGACAACG / ATCGTACTCCTGCTTGCTGATCCAC	60
ENT-1	TCATGCGAAAGCACCGAG / GGCACAGATCATGGCAAC	57
Myf-5	TGTATCCCTCACCAAGAGGAT / GGCTGTAATAGTTCTCCACCTGTT	55
EF-2	GACATCACCAAGGGTGTGCAG / GCAGTCAGCACACTGGCATA	62
BAMBI	TGTCCTCTCCTCCCAAGA / TGTCCATGGAAGCTGTAGTG	60
MSTN (qPCR)	GGCCATGATCTGCTGTAAC / TTGGGTGCGATAATCCAGTC	59
FS (qPCR)	AATGCCTACTGTGTACCTGT / GGCTCATCCGACTTACTGT	59
FSTL-3 (qPCR)	TGGTCTGGACAGTGGGATCCGT / TCCCGGCTGACACGAGTCTT	59
IGF-I	GGACCAGAGACCCTTGCGGGG / GGCTGCTTTGTAGGCTTCAGTGG	55
IGF-II	CCTTCGCCTTGTGCTGCAT / ACGGTTGGCACGGCTTGAA	55
IGF1R	CTGCGCGATGAAGAGAAGAAAA / TACCGGTGCCACGTTATGATGATT	55
IGFBP-3	AATGGCCCGGGTTCTGC / TTCTGGGTGTCTGTGCTTGAG	55
IGFBP-5	TGCCTAACGAAAAGAGCTACG / ACAAACTGGACTGGTCAGC	55
GAPDH (qPCR)	GACCCCTTCATTGACCTAAC / GATGACCTGCCACAGCCTT	59

primer set to assure specificity. In addition, amplicons were isolated on a 1% agarose gel to check for genomic DNA contamination or non-specific amplification. A master mix containing iQ Super mix (Bio-rad) was aliquoted before addition of cDNA and these samples were subsequently divided in half before primers were added. Samples were run in triplicate and each analysis was duplicated. Relative gene expression of ENT-1 was, therefore, normalized to that of the EF-2 using the Q-Gene method (Muller et al., 2002). Similar assays were performed on mouse heart cDNA using gene specific primers for myostatin (Shao et al., 2007), FS (Farnworth et al., 2006), GAPDH (Drummond et al., 2002), FSTL-3 (Table 2), IGF-I (Nagata et al., 2007), IGF-II (Montarras et al., 1996), IGF-1 receptor (Kim et al., 2008), IGFBP-3 (Kim et al., 2008) and IGFBP-5 (Caton et al., 2005). The samples were amplified at 94°C for 30 s, followed by 59°C for 30 s and 72 °C for 30 s using primer concentrations that were empirically determined (myostatin FP = 900 nM, RP = 900 nM; FS FP = 600 nM, RP = 900 nM; GAPDH FP = 900nM, RP = 900 nM; FSTL-3 FP = 600, RP = 900).

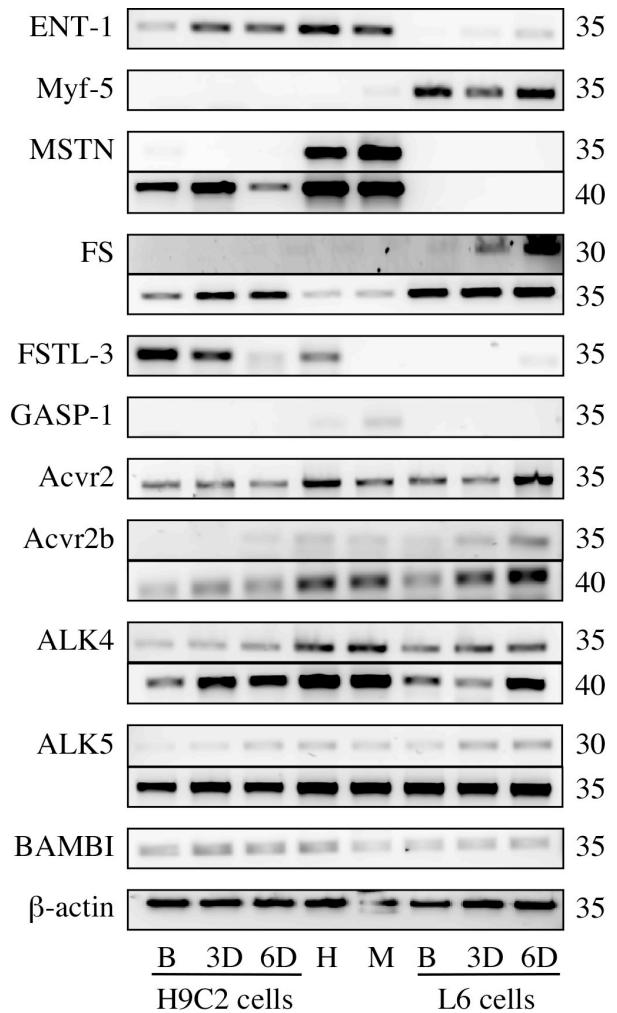
*Assessment of cardiac function and mass.* Mice were anesthetized in a closed system chamber by delivering 2.5% isoflurane in oxygen and maintained for the duration of the procedure with a nasal cone delivering 1% isoflurane. Standard imaging planes, M-mode, Doppler and functional calculations were obtained according to American Society of Echocardiography guidelines using the Acuson C512 echocardiography system (Siemens Medical Solutions Inc., [www.medical.siemens.com](http://www.medical.siemens.com)) with a 15L8 MHz epicardial transducer. The LV parasternal long axis 4-chamber view was used to derive ejection fraction (%EF) as well as ventricular dimensions and volumes. The left parasternal short axis view was used to obtain M-mode ventricular wall measures. The subcostal long axis view from the left apex was used

for Doppler imaging of mitral inflow and aortic ejection profiles. After a baseline echocardiogram was obtained for each mouse, a stress test was performed by injecting 10 mg/kg isoproterenol (Isuprel-R, Abbott Laboratories, [www.abbott.com](http://www.abbott.com)) intraperitoneally. Echocardiograph measurements were then recollected 3 minutes after injection. To independently validate the echocardial estimates of heart mass, body weight and body size, adult mice were also anesthetized with a cocktail of 60 mg/g ketamine, 50 mg/g pentobarbital and 0.2 U/g heparin, weighed and sacrificed by aortic puncture at seven months of age. Hearts were then removed and weighed, and tibias were removed and stained according to an adjusted protocol from (Greenspan et al., 1949). Heart and body weights were also measured in neonatal mice (<1 d.o.) killed by asphyxiation with CO<sub>2</sub>.

*Statistical analysis.* Three independent experiments were performed for the proliferation assays and two for the differentiation assays. Differences between means were determined by a 1- or 2-way analysis of variance coupled to Fisher's PLSD test or by a student's t-test when appropriate ( $p \leq 0.05$ ). Difference between regression curves of heart, body weight and body size over time were determined by a F-test ( $p \leq 0.05$ ).

## Results

*Expression of myostatin, its binding proteins and its receptors.* Qualitative expression analysis was performed in adult rat skeletal and cardiac muscle, proliferating H9C2 and L6 cells and in cells differentiating for 3 or 6 days (Fig. 2). Expression of ENT-1 and Myf-5 were used as differentiation and tissue-specific markers for cardiac and skeletal muscle, respectively, and changed as expected with differentiation. Myostatin expression was detected in proliferating

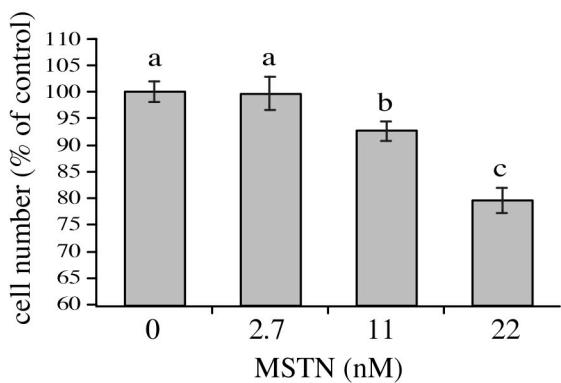


**Figure 2. Qualitative expression of myostatin, its binding proteins and its receptors during differentiation.** Equilibrative nucleoside transporter (ENT)-1 and myogenic factor (Myf)-5 were used as differentiation markers and  $\beta$ -actin as a loading control. Transcripts for myostatin (MSTN), follistatin (FS), follistatin-like (FSTL)-3, growth and differentiation factor associated protein (GASP)-1, activin type II receptors (Acvr2 & Acvr2b), activin like kinase (ALK4 & ALK5) and BMP & activin membrane-bound inhibitor (BAMBI) were amplified using qualitative RT-PCR. (B, myoblasts; 3D & 6D, days in differentiation medium; H, adult rat heart; M, adult rat skeletal muscle; H9C2, cardiomyoblasts; L6, skeletal myoblasts; cycle number indicated on the right).

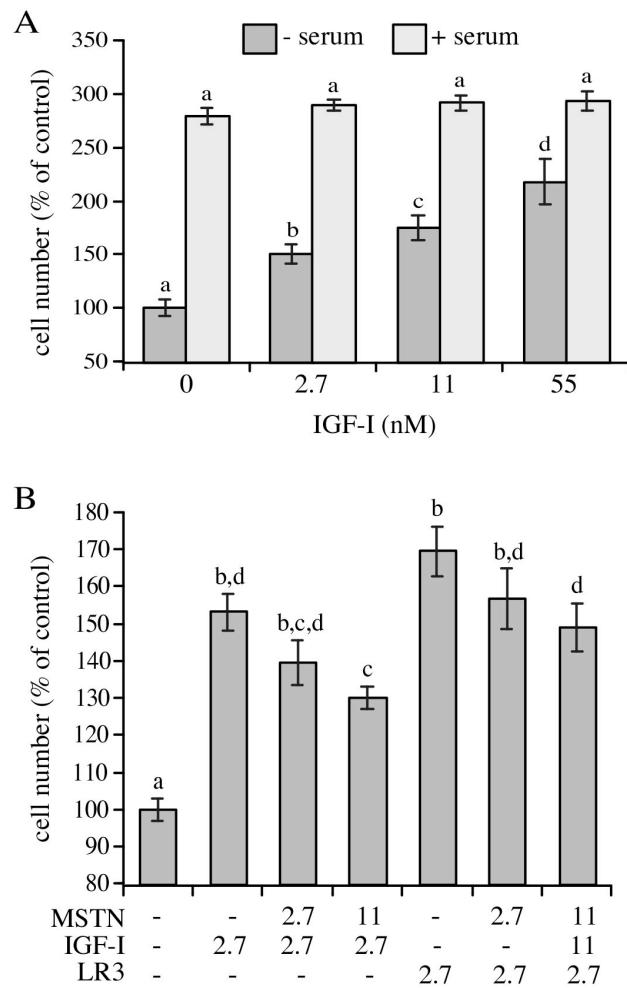
H9C2 cells, but not in L6 cells (even after 40 cycles - data not shown), and decreased with differentiation. Expression of the different myostatin binding proteins also differed in the H9C2 and L6 cells, which was consistent with the differences in cardiac and skeletal muscle expression. Follistatin expression was higher in L6 cells, increased with differentiation and was similar in adult cardiac and skeletal muscle. By contrast, significant expression of FSTL-3 only occurred in cardiac tissue and decreased in differentiating H9C2 cells while GASP-1 expression was only detected in mature skeletal muscle.

Expression of the different receptors was similar in both cell types, with the exception of Acvr2 expression as it increased in differentiating L6 cells, but not in H9C2 cells. Its expression was also detected five cycles earlier than that of Acvr2b. The lengths, calculated annealing temperatures and GC content of the primers used to amplify Acvr2 and Acvr2b message were identical. Therefore, the difference is likely not due to primer efficiencies, but to differences in mRNA levels. Similarly, expression levels of ALK-5 were also higher than those of ALK-4.

*Myostatin inhibits cardiomyoblast proliferation and differentiation.* H9C2 cells express all the important receptors necessary for myostatin signaling. Therefore, we determined if myostatin inhibits cardiomyoblast proliferation. Exogenous addition of myostatin significantly ( $p \leq 0.05$ ) inhibited basal proliferation of H9C2 cells in a dose-dependent manner when cells were cultured in serum free medium for 48 h (Fig. 3). Cell growth was inhibited by 8% and 21% with 11 and 22 nM MSTN, respectively, although the smaller dose of myostatin, 2.7 nM, did not have a significant effect.



**Figure 3. Myostatin inhibits basal cardiomyoblast proliferation.** H9C2 cells were cultured in serum-free media in the presence or absence of a range of myostatin (MSTN) concentrations. Cells were grown for 48 h before the total cell number was measured. Results from multiple experiments were pooled and data are expressed as % of controls. Significant differences ( $p \leq 0.05$ ) are indicated by different letters whereas the same letters denote no differences.

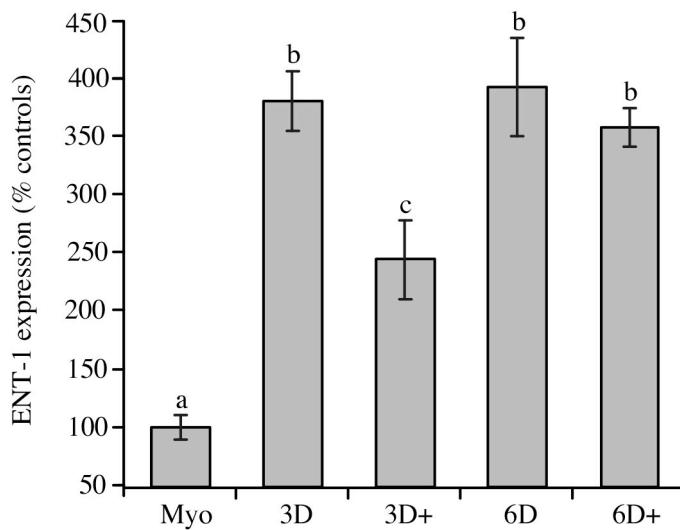


**Figure 4. Myostatin inhibits IGF-stimulated cardiomyoblast proliferation.** (A) H9C2 cells were cultured with IGF-I 48 h in the absence or presence of 10% serum (FBS). Results from multiple experiments were pooled by expressing data as % of controls for each time point. Significant differences ( $p \leq 0.05$ ) are indicated by different letters (comparisons within -/+ serum groups, not between groups) whereas the same letters denote no differences. (B) H9C2 cells were co-cultured with the indicated combinations and concentrations (nM) of myostatin (MSTN), IGF-I or LR3 in serum free medium. Cells were grown for 48 h before total cell number was measured. Results from multiple experiments were pooled and data are expressed as percent of controls. Significant differences ( $p \leq 0.05$ ) are indicated by different letters whereas same letters denote no differences.

IGF-I is a potent regulator of skeletal muscle growth and a determinant of animal size. It also stimulates proliferation of skeletal muscle cells (Adams, 2002). Addition of IGF-I stimulated the proliferation of H9C2 cells at 24 h and 48 h (Fig. 4A). This proliferative effect was only observed in serum free medium, but was dose-dependent with a minimum dose of 2.7nM significantly increasing total cell number. It has been reported that IGF binding proteins mediate the actions of myostatin in porcine myogenic cells (23-26). Therefore, we examined whether myostatin inhibits the actions of IGF-I or LR3, an IGF-I analog that does not bind IGF binding proteins (IGFBP), on cardiomyoblast proliferation. Myostatin significantly ( $p \leq 0.05$ ) suppressed the stimulatory effect of both IGF-I and LR3 and in a similar manner (Fig. 4B). The suppressive effects were dose-dependent as IGF-I stimulated growth was inhibited by 14 and 23 % with 2.7 and 11 nM myostatin, respectively. Myostatin had similar effects on LR3-stimulated proliferation (13 & 20%) suggesting that IGFBP sequestration of IGF-I is not involved.

Myostatin inhibits the differentiation of skeletal muscle cells by suppressing MyoD activity (Langley et al., 2002). Therefore, we examined myostatin's effect on the differentiation of cardiomyoblasts using quantitative expression of ENT-1 as a marker. The levels of ENT-1 mRNA increased more than 3-fold after 3 days in differentiation medium and remained constant thereafter (Fig. 5). The addition of 11 nM MSTN, however, partially suppressed ( $p \leq 0.05$ ) the rise in ENT expression. This suppressive effect was not observed after 6 days in differentiation medium, nevertheless, myostatin appears to inhibit the differentiation of cardiac as well as skeletal muscle cells.

*Myostatin influence on cardiac performance and mass.* To investigate the effect myostatin has on cardiac performance, echocardiography was performed on resting myostatin

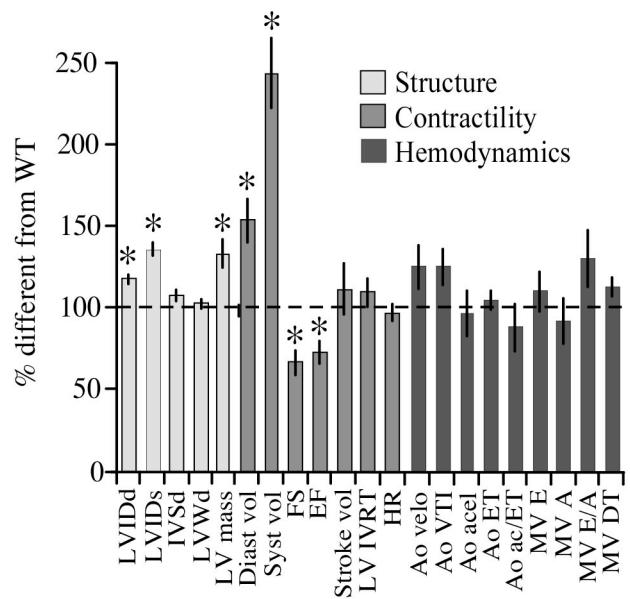


**Figure 5. Myostatin inhibits cardiomyoblast differentiation.** H9C2 cells were cultured in the presence or absence of 11 nM myostatin and stimulated to differentiate in 1% FBS and 10 nM retinoic acid added daily. Equilibrative nucleoside transporter (ENT)-1 was used as a differentiation marker and was quantified using “real-time” RT-PCR. (Myo, proliferating myoblasts; 3D/6D, days in differentiation medium; +, differentiation medium with 11 nM myostatin). Significant differences ( $p \leq 0.05$ ) are indicated by different letters, same letters denote no differences.

**Table 3. Cardiac performance of wild-type and myostatin null mice**

<b>parameter</b>	<b>WT</b>	<b>WT + iso</b>	<b>null</b>	<b>null + iso</b>
LVIDd	30.2 +/- 1.4	25.2 +/- 0.5	35.0 +/- 0.9	29.3 +/- 1.1
LVIDs	20.3 +/- 1.1	11.7 +/- 0.4	27.3 0.8	16 +/- 1.1
IVSd	8.2 +/- 0.2	8.8 +/- 0.3	8.7 +/- 0.3	9.5 +/- 0.4
LVWd	8.7 +/- 0.4	9.2 +/- 0.4	8.8 +/- 0.3	10.3 +/- 0.3
Diast vol	0.07 +/- 0.01	0.042 +/- 0.001	0.110 +/- 0.009	0.065 +/- 0.006
Syst vol	0.022 +/- 0.004	0.007 +/- 0.001	0.053 +/- 0.005	0.009 +/- 0.001
FS	33.1 +/- 1.0	53.6 +/- 1.4	21.5 +/- 2.5	46.75 +/- 2.4
EF	68.8 +/- 1.3	89.2 +/- 0.9	49.3 +/- 4.5	83.7 +/- 2.1
Stroke vol	0.050 +/- 0.006	0.037 +/- 0.002	0.055 +/- 0.008	0.055 +/- 0.006
LV IVRT	23.0 +/- 1.5	15.2 +/- 1.0	24.8 +/- 2.1	17.0 +/- 0.8
Ao velo	83.3 +/- 7.8	117.7 +/- 11.0	103.2 +/- 11.1	164.8 +/- 12.6
Ao VTI	3.4 +/- 0.5	5.2 +/- 0.4	4.3 +/- 0.4	6.3 +/- 0.6
Ao acel	14.2 +/- 1.7	5.8 +/- 0.5	13.7 +/- 1.9	10.2 +/- 1.4
Ao ET	63.3 +/- 3.4	69.2 +/- 3.1	65.6 +/- 3.5	62.8 +/- 3.7
Ao ac/ET	0.24 +/- 0.3	0.07 +/- 0.1	0.21 +/- 0.03	0.16 +/- 0.02
MV E	84.7 +/- 11.5	N.A.	92.3 +/- 10.6	121 +/- 8.1 (n = 3)
MV A	59.8 +/- 4.9	147.8 +/- 8.6	54.5 +/- 8.1	136 +/- 16.5
MV E/A	1.4 +/- 0.2	N.A.	1.8 +/- 0.2	1.2 +/- 0.2 (n = 3)
MV DT	25.5 +/- 1.8	N.A.	28.5 +/- 1.5	26 +/- 0.6 (n = 3)
LV mass	0.080 +/- 0.005	---	0.106 +/- 0.007	---
HR	361.5 +/- 9.8	443.4 +/- 18.4	346.7 +/- 17.8	460.3 +/- 16.8

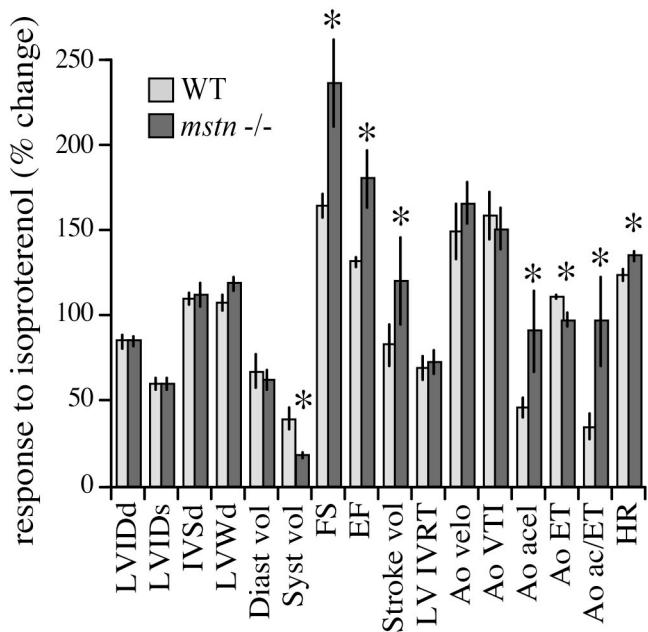
WT, wild-type *mstn*<sup>+/+</sup> mice; null, *mstn*<sup>-/-</sup> mice; iso, isoproterenol; LVIDd, left ventricle internal diameter (end diastole, mm); LVIDs, LVID systole; IVSd, intraventricular septum (dimension end diastole, mm); LVWd, LV wall dimension (systole, mm); Diast vol, LV end diastolic volume (ml); Syst vol, LV end systolic volume (ml); FS, % fractional shortening; EF, % ejection fraction; LV IVRT, LV isovolumic relaxation time (ms); Ao velo, max aortic ejection velocity (cm/s); VTI, velocity time intergral (cm); acel, ejection acceleration time (ms); ET, ejection time (ms); ac/ET, ratio of acel to ET; MV E, max LV early filling velocity (cm/s); A, max LV late filling (atrial contraction) velocity (cm/s); E/A, ratio of E to A velocities; DT, deceleration time of early LV filling (ms); LV mass, left ventricle mass (g); HR, heart rate (b/m); N.A., not available; different letters denote statistical differences within a single measured parameter



**Figure 6. Enhanced resting cardiac performance in myostatin null mice.** Echocardiography was performed on the LV parasternal long axis, left parasternal short axis and subcostal long axis views. Data are presented as percent different from wild type (WT) values represented by the horizontal dashed line (LVIDd, left ventricle internal diameter (end diastole, mm); LVIDs, LVID systole; IVSd, intraventricular septum (dimension end diastole, mm); LVWd, LV wall dimension (systole, mm); Diast vol, LV end diastolic volume (ml); Syst vol, LV end systolic volume (ml); FS, % fractional shortening; EF, % ejection fraction; LV IVRT, LV isovolumic relaxation time (ms); Ao velo, max aortic ejection velocity (cm/s); VTI, velocity time integral (cm); acel, ejection acceleration time (ms); ET, ejection time (ms); ac/ET, ratio of acel to ET; MV E, max LV early filling velocity (cm/s); A, max LV late filling (atrial contraction) velocity (cm/s); E/A, ratio of E to A velocities; DT, deceleration time of early LV filling (ms); LV mass, left ventricle mass (g); HR, heart rate (b/m).) Structure, contractility and hemodynamic parameters are grouped and are indicated by differential shading and asterisks denote significant differences ( $p \leq 0.05$ ) from WT.

null and wild-type mice (Table 3 & Fig. 6). Left ventricular mass was significantly larger in the null mice as were the left ventricle internal diameters at diastole and systole (LVIDd & LVIDs), although intraventricular septum (IVSd) and (IVWd) wall diameters were similar in both groups. This indicates that the increased heart size in myostatin null mice is due to eccentric, not concentric, hypertrophy. Diastolic and systolic volumes were also significantly increased in myostatin null hearts, which is consistent with eccentric hypertrophy. Fractional shortening (FS) and ejection fraction (EF), however, were significantly decreased, although stroke volume was similar in null and wild-type mice. Measurements of aortic hemodynamics and left ventricle filling were also similar.

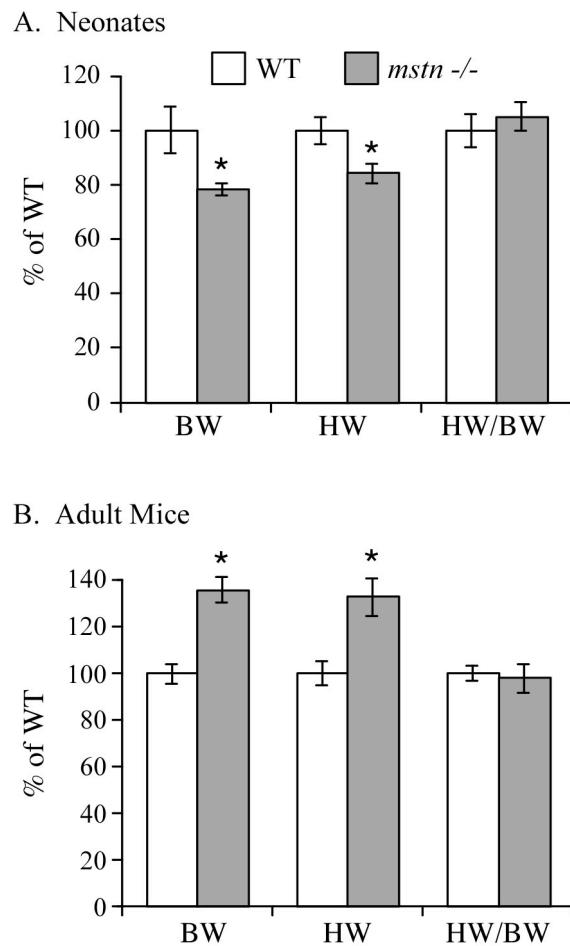
Cardiac stress tests were also performed by administering isoproterenol, a  $\beta$ -adrenergic receptor agonist (Table 3 & Fig. 7). The percent change in internal diameters and diastolic volume was similar in both groups despite absolute differences attributed to the eccentric hypertrophy in null mice (Table 3). However, the percent change in systolic volume was significantly less in myostatin null mice and was accompanied by greater increases in fractional shortening and ejection fraction. As expected, isoproterenol-induced tachycardia reduced stroke volume in wild-type mice, although by contrast, stroke volume actually increased in null mice. Thus, stress-induced relaxation and contractile capacity and cardiac output were both increased in null mice at similar heart rates. As a consequence, the change in aortic acceleration (Ao acel) was greater in null mice and resulted in a shorter ejection time (Ao ET) and increased acel/ET ratio. The change in heart rate was also greater in null mice and likely contributed to the hemodynamic differences. The increased heart rate in response to isoproterenol prevented comparisons of left ventricle filling parameters, which often occurs when performing stress tests



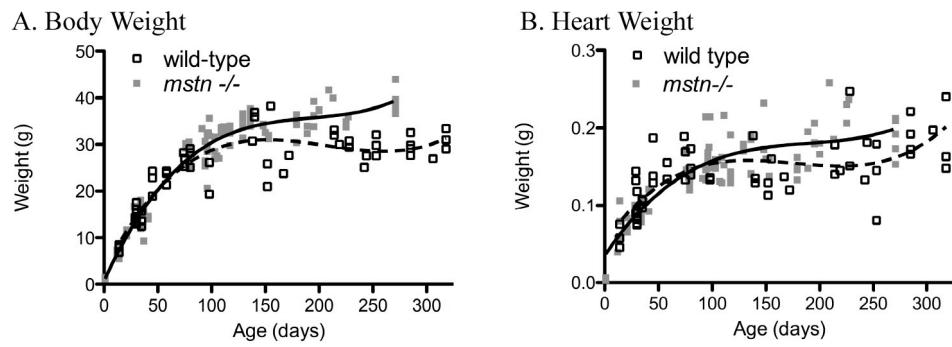
**Figure 7. Enhanced cardiac stress response in myostatin null mice.** Echocardiography was performed on mice injected intraperitoneally with 10 mg/kg isoproterenol using LV parasternal long axis, left parasternal short axis and subcostal long axis views. Data are represented as percent change from resting values (before isoproterenol treatment). Acronyms are defined in the Figure 5 legend and asterisks denote significant differences ( $p \leq 0.05$ ).

on mice. These parameters, however, were distinguished in some or all of the null mice (Table 3) possibly due to longer diastolic filling times.

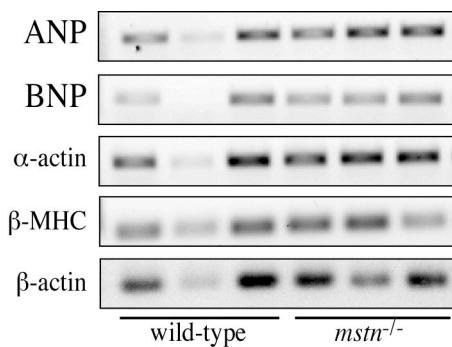
Heart weights were also measured manually in adult mice and in neonates. Both heart and body weights of the wild-type neonates were slightly larger than those of myostatin null mice (Fig. 8A), although the heart weight to body weight (HW/BW) ratios were comparable in both groups (Fig. 8A). By contrast, adult hearts from null mice were almost 33 % heavier than those from wild-type mice (Fig. 8B), which was similar to the differences in body weights. In fact, the HW/BW ratios were nearly identical in both groups, suggesting that similar mechanisms may be responsible for both phenotypes. Additionally, heart and body weights were measured over time, from one day through seven months of age (Fig. 9). Body weights of myostatin null and wild type mice begin diverging at about three months of age, and while wild type body weight plateaus shortly thereafter, myostatin null body weights continue to increase through seven months of age (Fig 9A). Heart weights follow a similar trend, with myostatin null and wild type heart rates diverging significantly only after three months, after which wild type mice heart weights plateau while myostatin null heart weights continuing to increase (Fig. 9B). Cardiac hypertrophy can result from pathological or physiological means (Catalucci et al., 2008; McMullen and Jennings, 2007). The former is associated with an upregulation of genes normally expressed during fetal development, such as ANP, BNP,  $\alpha$ -actin and  $\beta$ -MHC. We therefore surveyed the expression of these genes in myostatin null and wild-type mice, but found no differences in expression between the two mouse groups (Fig. 10). Therefore, the fetal gene expression pattern that commonly occurs with pathological hypertrophy of the heart does not occur in hearts of myostatin null mice.



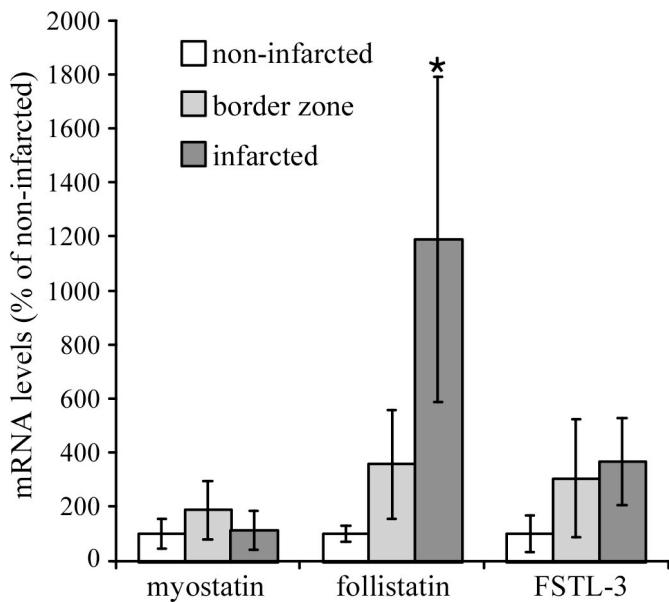
**Figure 8. Heart and body weight relationships in neonatal and adult mice.** Heart weight (HW), body weight (BW) and HW/BW ratio of wild type and myostatin null neonates (A) and adult (B) mice. Data are expressed as a percent of wild type values. Asterisks denote significant difference ( $p \leq 0.05$ ).



**Figure 9. Body and heart weight over time in myostatin null and wild type mice.** Open squares and dashed lines denote wild type values, solid squares and solid lines denote myostatin null values. (A) Body weight of myostatin null and wild type mice over 300 days. (B) Heart weight of myostatin null and wild type mice over the course of 300 days.



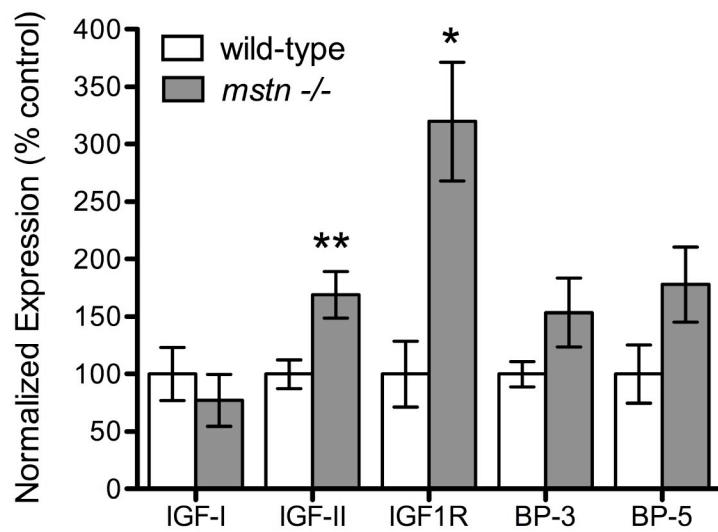
**Figure 10. Fetal gene expression profile.** Cardiac transcripts for atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP),  $\alpha$ -actin, b-myosin heavy chain (MHC) and  $\beta$ -actin (cDNA quality control) were amplified for 30 cycles by RT-PCR. Samples from three represented wild-type and myostatin null ( $mstn^{-/-}$ ) mice are shown.



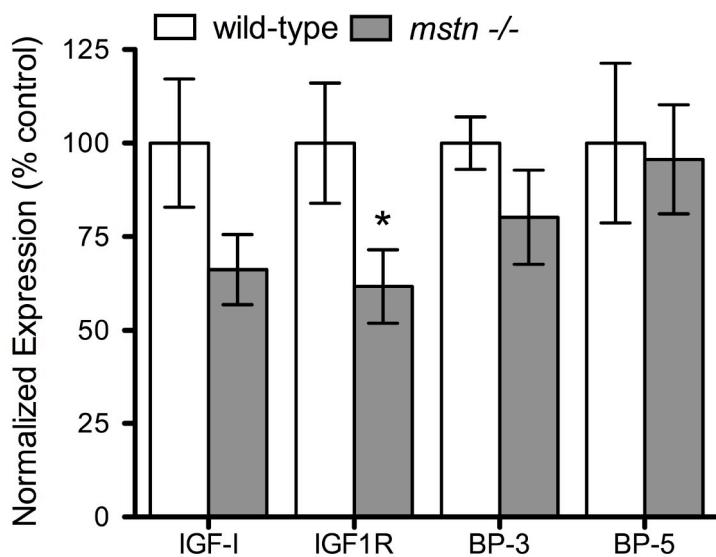
**Figure 11. Quantitative expression analysis of myostatin and its binding proteins in recovering cardiac muscle.** Myocardial infarctions were induced as described in the Materials and Methods and non-infarcted, border zone and infarcted cardiac muscle was removed after 28 days. Expression of myostatin, follistatin, and follistatin-like (FSTL)-3 was quantified using gene-specific “real time” PCR. Asterisk denotes significant differences from non-infarcted values ( $p \leq 0.05$ ).

*Cardiac expression of myostatin and its binding proteins following an infarct.* To help determine myostatin's role in regenerating cardiac muscle, the expression of myostatin, follistatin and FSTL-3 was measured in infarcted, border-infarcted and non-infarcted cardiac muscle 28 days after ischemia-reperfusion injury. Normalized expression levels for all three genes were highly variable (Fig. 11), especially in the border-infarcted and infarcted muscle. Myostatin expression was therefore similar in the different regions. Despite this variability, however, expression of follistatin and FSTL-3 were highly elevated in the affected tissues, although these differences were only significant within the infarcted muscle.

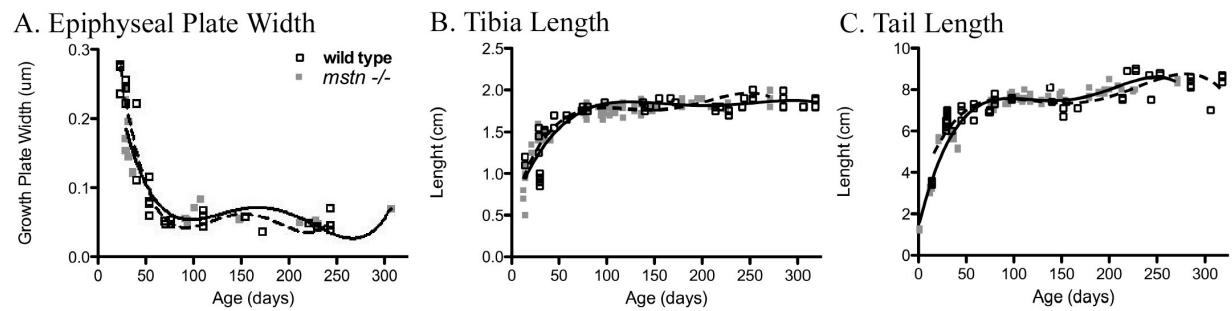
*The IGF axis's effect on body size and heart weight.* The IGF-I/IGFBP axis stimulates cellular growth in an endocrine and autocrine fashion (Jones and Clemons, 1995). Early reports from our lab indicate that expression of IGF-I and its interacting partners are altered in skeletal muscle of myostatin knockout models (data not shown), so we investigated the expression of IGF-I, -II, IGF-I receptor (IGF-IR), and IGFBP-3 and -5 in the cardiac muscle of myostatin null and wild type mice. In neonatal mice, IGF-II, and IGF-IR were significantly elevated by approximately 70% and 250%, respectively (Fig 12), whereas in adult mice IGF-IR was decreased (40%) (Fig 13). The expression of IGF axis partners, which control cardiac muscle growth (Lombardi et al., 1997) and overall body size are therefore differentially regulated by myostatin in neonatal versus adult mice. Changes in IGF/IGFBP production could presumably affect other tissues as well. Thus, we investigated the effect of myostatin knockout on body size over a period of seven months by measuring tibia epiphyseal plate width (Fig. 14A), tibia length (Fig. 14B), and tail length (Fig. 14C). However, there was no significant difference in these parameters between myostatin null and wild type mice at any time period.



**Figure 12. Expression of IGF-I, IGF-II, their receptor and binding proteins in cardiac tissue of neonatal myostatin null and wild type cardiac muscle.** Hearts were removed as described in Methods and Materials. Expression of IGF-I, IGF-II, IGF-I receptor (IGF1R), IGFBP-3, and IGFBP-5 was quantified using gene-specific “real time” PCR. \*\* denotes significant differences from control values where  $p \leq 0.001$ , \* denotes significant differences from control values where  $p \leq 0.05$ .



**Figure 13. Expression of IGF-I, IGF-II, receptor and binding proteins in adult myostatin null and wild type cardiac muscle.** Hearts were removed as described in Methods and Materials. Expression of IGF-I, IGF-I receptor (IGF1R), IGFBP-3, and IGFBP-5 was quantified using gene-specific “real time” PCR. Asterisk denotes significant differences from control values ( $p \leq 0.05$ ).



**Figure 14. Body size measurements over time in myostatin null and wild type mice. (A)**

Tibia epiphyseal plate width was stained and measured as described in Methods and Materials.

(A-C) Open squares and dashed lines denote wild type values, solid squares and solid lines denote myostatin null values. (A) Width of epiphyseal plate for myostatin null and wild type mice were determined for mice aged one week through 300 days. (B) Tibia length was measured in myostatin null and wild type mice over the course of 300 days. (C) Tail length was measured in myostatin null and wild type mice over the course of 300 days.

This indicates that increased body weight in myostatin null mice is due only to increased muscle size and that changes in the cardiac production of IGF/IGFBP axis components likely influences this tissue alone.

### ***Discussion***

Myostatin's negative influence on the growth and development of skeletal muscle, both *in vitro* and *in vivo*, is well documented and has been described to varying degrees in different mammalian model systems (Rodgers and Garikipati, 2008). The lack of an equally well established cardiac phenotype among the different null animals is somewhat surprising as several studies have at least implied functional roles for myostatin in cardiac muscle (Sharma et al., 1999; Shyu et al., 2005; Shyu et al., 2006). Indeed, the mass of both skeletal and cardiac muscle is reduced in transgenic mice overexpressing myostatin (Reisz-Porszasz et al., 2003) and together with the current study, other recent studies have clearly demonstrated myostatin's ability to inhibit some cardiac muscle growth processes (McKoy et al., 2007; Artaza et al., 2007; Morissette et al., 2006). Thus, myostatin appears to possess similar functional roles and is a proposed chalone in both muscle types (Gaussin and Depre, 2005; Lee, 2004).

Myostatin signals via activin receptors, specifically the type II receptors Acvr2 and Acvr2b (a.k.a. ActRIIa & ActRIIb), which recruit the type I receptors ALK-4 and -5 (Rodgers and Garikipati, 2008). The active receptor complex phosphorylates Smad 2 and 3 and can theoretically be perturbed by BAMBI, a pseudoreceptor that sequesters ALK-4/5 and prevents activation (Onichtchouk et al., 1999; Sasaki et al., 2004; Sekiya et al., 2004). We determined that cardiac muscle and H9C2 cells express all of these receptors (Fig. 2) as well as two notable

myostatin binding proteins, follistatin and FSTL-3, but not GASP-1. Thus, proliferating, differentiating and mature cardiac muscle cells should be responsive to myostatin and additionally possess the capability to manipulate myostatin bioavailability. Levels of Acvr2 expression were higher than those of Acvr2b in all the samples (Fig. 2). A similar observation was also observed in different mouse skeletal muscles (Lee et al., 2005). In fact, the muscle mass of transgenic mice overexpressing dominant-negative Acvr2 is greater than that of similar mice overexpressing dominant-negative Acvr2b (Lee et al., 2005). This suggests that although myostatin is capable of signaling through both receptors, Acvr2 may play a more important role in regulating skeletal and/or cardiac muscle growth especially as binding assays indicate that myostatin's affinity for Acvr2b is estimated to be only slightly higher than it is for Acvr2 (Lee and McPherron, 2001).

Levels of myostatin expression are high in the developing mammalian fetal heart, but are considerably lower in adult hearts (Sharma et al., 1999). In another study (McKoy et al., 2007), myostatin mRNA and protein levels increased in cardiomyocytes from embryonic day 18 to 10 days after birth when proliferation stops (Poolman and Brooks, 1998). These patterns of expression were reflected in differentiating H9C2 cells (Fig. 2), which further validates the model, and are consistent with myostatin's expected role as a cardiac muscle differentiation factor. Indeed, we determined that myostatin inhibits basal and IGF-stimulated cardiomyoblast proliferation (Figs. 3 & 4). Similar results were recently reported in studies using primary fetal and neonatal cardiomyoblasts (McKoy et al., 2007) and in H9C2 cells (Artaza et al., 2007), although myostatin's effects on IGF-stimulated proliferation were not examined. Growth inhibition in these studies was accompanied by G1 cell cycle arrest, a decrease in cyclin-

dependent kinase (Cdk) 2, an increase in p21 (a Cdk2 inhibitor) and phosphorylation/activation of Smad2. All of these studies, including the current, together suggest that myostatin's inhibitory effects in cardiomyoblasts are mechanistically similar to those in skeletal muscle cells (McCroskery et al., 2003; Taylor et al., 2001; Thomas et al., 2000; Rios et al., 2001).

Insulin-like growth factor-I is a potent mitogen for many cell types (LeRoith and Roberts, 2003; Dayton and White, 2007), although its proliferative effects in cardiomyoblasts, particularly the H9C2 cell line, has not been well documented. It stimulated H9C2 cell proliferation in a dose dependent manner (Fig. 4) and these effects were suppressed by myostatin whether IGF-I or the LR3 analog were used. Myostatin's inhibitory effects in porcine myogenic cells are mediated in part by the local production of IGFBP-3 and -5 (Kamanga-Sollo et al., 2003; Kamanga-Sollo et al., 2005; Pampusch et al., 2005; Xi et al., 2006), which sequester IGF-I outside the cell and may also inhibit intracellular or nuclear actions required for cell growth (Oufattolle et al., 2006; Xi et al., 2007). However, IGFBPs do not appear to mediate myostatin's actions in H9C2 cells as it suppressed the proliferative effects of both IGF-I and LR3 equally. Myostatin has also been shown to suppress IGF- and phenylephrine-stimulated protein synthesis in fully differentiated cardiomyocytes (Shyu et al., 2005; McKoy et al., 2007; Morissette et al., 2006). Its inhibitory effects on IGF action and signaling in cardiac muscle cells are, therefore, not limited to differentiation status. Our results also indicate that myostatin at least partially suppresses the differentiation of H9C2 cardiomyoblasts (Fig. 5). Expression of ENT-1 in treated cells was approximately half that in control cells after 3 days in differentiation medium, although these effects were lost by day 6. Myostatin was only added on days 1 and 3, so the suppressive effect on differentiation was likely nullified in part by daily stimulation with retinoic

acid. Nevertheless, ENT-1 expression and cardiomyoblast differentiation were clearly inhibited by myostatin after 3 days. Thus, myostatin inhibits all of the processes required for the development of mature cardiac muscle: proliferation, differentiation and protein synthesis.

Previous studies have disagreed on the cardiac phenotype of myostatin null animals. Cohn *et al.* (Cohn et al., 2007) reported no differences in ventricular mass, heart weight, HW/BW ratio or cardiomyocyte area of myostatin null and wild-type mice. By contrast, Artaza *et al.* (Artaza et al., 2007) reported increases in heart mass and left ventricle size in myostatin null mice. We therefore reexamined the cardiac phenotype by assessing differences in heart mass and cardiac performance in myostatin null and age-matched wild-type mice using echocardiography. Assays were performed on resting hearts (Table 3 & Fig. 6) and for the first time on hearts stimulated with isoproterenol (Table 3 & Fig. 7). Our results indicate that adult myostatin null mice have significantly larger hearts that are approximately 33 % heavier. These results were corroborated by physically weighing the hearts and by echocardiographical estimates of left ventricle mass, both of which are proportional. This parallel increase in heart weight and body weight suggests that similar mechanisms may control both phenotypes. This is supported by the previously discussed direct and negative effects of myostatin on different cardiac muscle growth processes. The heart and body weights of neonatal myostatin null mice were slightly less than those of wild-type pups. Thus, cardiac hypertrophy in myostatin null mice develops postnatally or is at least an ontological consequence, but is not due entirely to enhanced development *in utero*.

Measurements of internal diameter, but not wall thickness, were increased in null mice indicating eccentric as opposed to concentric hypertrophy. Myostatin null mice had significantly

lower systolic contraction functions at rest, as measured by fractional shortening and ejection fraction, but moved similar volumes of blood and possessed a heightened response to  $\beta$ -adrenergic stimulation, which included enhanced contractility and hemodynamic measurements. These results are consistent with the preliminary results of Artaza *et al.* and provide a far more thorough assessment of cardiac function in myostatin null animals than previously reported. They also suggest that myostatin regulation of cardiac muscle development directly influences cardiac performance at rest and during a stress test. Cohn *et al.* used 24 month old mice. It is therefore difficult to determine whether age was somehow a confounding factor. In fact, they report identical body weights for both wild-type and myostatin null mice, which conflicts with the latter group's well documented double muscled phenotype (McPherron *et al.*, 1997). Our studies of heart and body weight over time (Fig. 9) definitively show that myostatin null mice have significantly larger skeletal muscle and hearts than wild type mice and that these weights diverge from wild type mice at the same age.. However, despite changes in the expression of IGF axis components in neonatal and adult myostatin null mice (Fig. 12, 13), there appears to be no correlated increase in bone growth, as measured by tail length, tibia length, or tibia epiphyseal growth plate width (Fig. 14). Interestingly, both body weight and heart weight continued to increase over the lifetime of the myostatin null mice, well into the seventh month of age and long after growth in wild type mice stopped. A similar pattern was observed in transgenic mice overexpressing the LAP who were fed a high fat diet and had an increase in skeletal muscle weight up to 30% greater than transgenic mice on a normal diet (Yang and Zhao, 2006).

Cardiac hypertrophy, simply defined as an increase in heart mass, can be a significant contributor to heart failure and is associated with a number of cardiovascular pathologies

including hypertension and myocardial infarctions. Hypertrophy *per se*, however, is not necessarily bad as it can arise from either pathological or physiological means, of which the latter is unquestionably beneficial. The two phenotypes are molecularly and functionally distinct as pathological hypertrophy is generally associated with contractility disorders, reduced cardiac output, especially during a stress test, and the expression of fetal genes (ANP, BNP, a-actin & b-MHC) in contrast to physiological hypertrophy (Catalucci et al., 2008; McMullen and Jennings, 2007). Hearts from myostatin null mice were bigger and for the most part functionally superior to those of wild-type mice. This level of eccentric hypertrophy and enhanced cardiac performance is comparable to that of highly trained endurance athletes (Stout, 2008; Li et al., 2008). Myostatin null hearts also lack the fetal gene expression profile (Fig. 10) and increased fibrosis (Cohn et al., 2007) that occurs with pathological hypertrophy. In fact, myostatin was recently demonstrated to stimulate fibroblast proliferation *in vitro* and to increase skeletal muscle fibrosis *in vivo* (Li et al., 2008). Thus, the myostatin null mouse is a potentially novel and unexplored model of physiological hypertrophy.

The cardiac stress tests revealed a heightened response to catecholamines in myostatin null mice as many cardiac systolic function and hemodynamic responses to isoproterenol were greater in null than in wild-type mice (Fig. 7). This positively influenced cardiac performance, which is possibly best illustrated by the maintenance of stroke volume in null, but not wild-type mice. Thus, cardiac output was maintained in null mice despite the reduction in LV filling time that occurs with increased heart rate. However, the heightened response was not simply due to physiological hypertrophy (i.e. larger, stronger hearts pumping more blood faster) as the heart rate response to isoproterenol was also faster in null mice. It is unclear whether the increased

sensitivity to isoproterenol is due to enhanced b-adrenergic receptor expression, binding kinetics or intracellular signaling. These data do however suggest that myostatin and  $\beta$ -adrenergic signaling are at least acutely related and provide a potential mechanism by which myostatin may influence cardiac performance independent of its effects on cardiac muscle growth.

The percent fractional shortening (FS) and ejection fraction (EF) were proportionally lower in resting null mice (Fig. 6). Reductions in these parameters can, but do not necessarily, indicate pathological abnormalities of cardiac function. Indeed, they are usually unaltered in the hypertrophied heart regardless of cause (Stout, 2008). Furthermore, pathological hypertrophy is defined by an impaired EF during a stress test, which is opposite from the heightened response seen in myostatin null mice (Fig. 7). A subset of highly trained athletes, particularly those that combine endurance and strength training, have decreased resting EF values that rise to normal with detraining (Pavlik et al., 1986; Abergel et al., 2004). This is likely due to reduced preload in the presence of normal or enhanced afterload and contractility (Colan, 1997). Nevertheless, this further illustrates the utility of the myostatin null mouse for studying the mechanisms responsible for physiological hypertrophy.

Skeletal muscle expression of myostatin mRNA and/or protein increases with various insults and is believed to contribute to the repair process possibly by maintaining myosatellite cell quiescence throughout the differentiation cycle (Rodgers and Garikipati, 2008). Its expression similarly increases in hypertrophied or infarcted cardiac muscle, although it is unknown whether the expression of myostatin binding proteins similarly changes and potentially influences myostatin bioavailability. We therefore measured the mRNA levels of follistatin and FSTL-3 in cardiac muscle extracted from mice recovering from experimentally induced

myocardial infarctions. Mean expression levels of both genes were higher in the infarcted and border zones than in the non-infarcted zone (Fig. 11), although the only statistically significant difference occurred with follistatin expression. Myostatin expression was similar in all tissues, which was not necessarily surprising considering the large variability among samples. Whether or not changes in myostatin, follistatin or FSTL-3 expression influence the development of pathological hypertrophy following an infarct remains to be determined. These results nevertheless suggest that regenerating cardiac muscle possesses the capacity to disrupt myostatin bioavailability through the local expression of myostatin binding proteins.

## **CHAPTER 3**

### **IMPLICATIONS OF CONTROLLING MYOSTATIN FUNCTION**

Disrupting myostatin bioactivity and/or availability may potentially impact the treatment of many clinical disorders. Patients with some forms of muscular dystrophy or other skeletal muscle pathologies are obvious candidates for such novel therapeutics (Bogdanovich et al., 2002; Bogdanovich et al., 2007; Chakkalakal et al., 2005). Studies suggest that myostatin blockade could prove to be a treatment for cachexia and obesity, as well (McPherron and Lee, 2002; Yang and Zhao, 2006; Zhao et al., 2005). The data presented herein as well as the other recent studies (Artaza et al., 2007; Shyu et al., 2006) of myostatin action in cardiac muscle suggest that targeting myostatin may also help treat cardiac muscle growth disorders. Specifically, they suggest that myostatin blockade could help replace pathological hypertrophy of the heart with physiological hypertrophy and thus, improve cardiac muscle repair mechanisms as well as clinical outcomes for patients recovering from a myocardial infarction. These therapeutics could also benefit cell-based procedures that depend upon the generation of viable cardiomyocytes from embryonic or non-embryonic stem cells (Laflamme et al., 2007; Nomura et al., 2007). Nevertheless, future studies with myostatin null mice will no doubt help determine the underlying mechanisms responsible for the development of physiological hypertrophy of the heart.

#### ***Muscular Dystrophy***

The most severe form of muscular dystrophy, Duchenne's muscular dystrophy (DMD) presents with weakness and muscle wasting and is detectable by age two or three (Prior and

Bridgeman, 2005). It results from an X-linked mutation of the dystrophin gene and occurs in nearly 1 in 3500 males in the US, with many different varieties determined by the severity of the mutation (Prior and Bridgeman, 2005). The dystrophin protein stabilizes the contractile structures of striated muscle; null mutations in this protein result in muscle weakness, muscle deterioration, and eventual fibrosis of both skeletal and cardiac muscle (McNally, 2007). In fact, many deaths from DMD are a result of cardiac failure. Studies have already investigated the possible use of myostatin blockade on DMD treatment, in the hopes that removing the control over muscle growth through upregulation of myostatin blocking proteins or inactivation of myostatin could ameliorate the muscle wasting symptoms (Bogdanovich et al., 2002; Bogdanovich et al., 2008; Bogdanovich et al., 2005; Byron et al., 2006; Cohn et al., 2007; Colussi et al., 2008; Nakatani et al., 2007; Parsons et al., 2006; Qiao et al., 2008; Wagner et al., 2002). The results as a whole show increased skeletal muscle cell size, reduced fibrosis, and improved muscle strength. Two studies also looked at cardiac tissue histology, however there was no reported cardiac hypertrophy (Cohn et al., 2007; Qiao et al., 2008). Further studies are required as the researchers were looking for pathological cardiac hypertrophy, not physiological hypertrophy.

### ***Obesity & Type II Diabetes Mellitus***

An even more prevalent health issue is obesity. Worldwide obesity rates are now believed to be at epidemic levels (Low et al., 2009). Obesity is undesirable not only for cosmetic purposes, but is associated with many other life-threatening conditions like diabetes, heart disease, and cancer (Low et al., 2009). Myostatin appears to play a role in fat deposition as preliminary studies of myostatin null mice had significantly reduced fat pad size (McPherron and

Lee, 2002; Yang et al., 2001). Additional studies found that myostatin null mice on a high-fat diet had increased body weights, but the increase was due to muscle growth and not fat deposition (Zhao et al., 2005). Insulin, leptin and resistin levels, all indicators of obesity, were also normal in these mice (Zhao et al., 2005). When crossed with agouti lethal yellow mice, which spontaneously develop type II diabetes, glucose tolerance was maintained and no signs of insulin resistance were detected (McPherron and Lee, 2002; Zhao et al., 2005). Thus, myostatin appears to positively regulate the development of obesity and type II diabetes.

These findings are especially critical as type II diabetes has become one of the greatest health risks in the US. Once labeled “adult-onset diabetes”, rates of occurrence are rising in children concurrent with obesity rates (Sherwin et al., 2004). This form of diabetes is marked by insulin insensitivity and high blood sugar levels, which has systemic deleterious effects that increase susceptibility to pneumonia, cause eyesight problems, stroke, and heart disease – health problems normally reserved for adults, indeed elderly, but now occur in children (Sherwin et al., 2004). The ability of myostatin blockade to inhibit insulin resistance is therefore very promising for the treatment of type II diabetes. Losing weight can ease or even cure type II diabetes (Anderson et al., 2003), so the decrease in fat deposition and increase in skeletal muscle resulting from myostatin blockade could alleviate or prevent type II diabetes.

### ***Heart Disease***

Another health risk associated with obesity and diabetes is heart disease. Sedentary lifestyles and poor eating habits have led to a prevalence of hypertension and dangerous cholesterol levels (Grundy et al., 1987). Changes in diet and exercise can often address these pathologies, but any doctor will tell you that these are almost impossible changes to implement

and maintain for most patients. The results reported in this paper show that myostatin null mice possess the physiological cardiac hypertrophy of well-trained athletes, even when not exposed to exercise training. Thus, further studies should investigate the feasibility of myostatin blockade as preventative treatment for hypertension; the ideal “all gain, no pain” exercise model.

Myostatin blockade could also be used to treat pathologies that have already damaged cardiac tissue, such as myocardial infarction which can produce chronic heart failure. Such injuries are especially dangerous to the heart as cardiac tissue does not have the stem cell population to replace damaged tissue. The current model of myocardial infarction and pathological hypertrophy is that after the initial injury, cardiac tissue tries to repair itself through cellular hypertrophy (Leferovich and Heber-Katz, 2002). This initially works, but due to the increased wall thickness and lack of proportional chamber increase coupled with increased fibrosis, the muscle is unable to properly function as contractile and hemodynamic parameters decrease, which eventually leads to heart failure (McMullen and Jennings, 2007; Catalucci et al., 2008). Myostatin blockade would presumably produce hypertrophy that leads to eccentric hypertrophy, like that of an athlete, and prevent or substantially delay heart failure. The results reported herein indicate that myostatin binding proteins increase in infarcted cardiac tissue – perhaps the body’s attempt to create a myostatin null environment for recovery. Myostatin blockade technologies could therefore complement this natural process.

### ***Therapeutics for Myostatin Blockade***

The majority of studies investigating myostatin have been implemented in rodent models or cell culture, with the aim to create a technology applicable to human use. Genetically manipulating mice and/or cells is easily accomplished using technologies that are not yet

advanced enough for human treatment. The most promising of these technologies, however, is adeno-associated viruses (AAVs), which can direct transgenes to specific tissues or broadly depending on the AAV serotype (Daya and Berns, 2008). Viral gene therapy has already entered clinical trials, but is plagued with controversy and bad press. Adenoviruses are particularly virulent vectors that cause a variety of diseases (Daya and Berns, 2008). Due to their carrying capacity and widespread availability, these were traditionally the virus of choice for animal gene therapy experiments. However, due to the extreme immune response elicited in humans, an alternative to adenoviruses had to be developed (Zaiss et al., 2002). Adeno-associated viruses are similar to adenoviruses in many ways except one – they have evolved with humans, and thus do no more than cause a common cold when introduced to the immune system (Daya and Berns, 2008). Clinical trials have begun using AAVs, but the use of immunosuppressants has resulted in unresolved clinical issues and controversy (Daya and Berns, 2008). Nevertheless, AAVs still present the most promising method of myostatin blockade using a gene therapy approach.

### ***Performance Enhancement***

One reason why investigating myostatin is so critical is that it has a high potential for abuse among athletes. As myostatin mutations produce a highly visual phenotype, e.g. the double muscling in myostatin knockouts like Belgian Blue cattle and the “bully” whippet (see Fig. 1), public knowledge grows along with the potential for abuse. Body builders seeking an untraceable way to increase muscle mass have already become targets for so-called myostatin blockers. These factors are isolated from seaweed, but have shown no demonstrable effect on myostatin action (Willoughby, 2004). Should myostatin blocking technologies reach the human level, their availability may cross the same ethical lines of conflict as anabolic steroids in

organized sports. Other ethical concerns include myostatin's ability to block fat deposition. The uncontrolled use of legitimate myostatin blockers for the pursuit of an absolute ideal body type could also produce deleterious side effects. Myostatin could well be touted as the miracle weight loss drug. Unlike the current alternative to get the ideal figure, plastic surgery, which has not shown any affect on insulin resistance or incidence of heart disease (Klein et al., 2004), myostatin blockade may improve these parameters. However, while current studies indicate myostatin blockade has no negative side effects, all possible implications are as yet unclear. If not fully studied and understood, myostatin blocks could indeed become the next designer drug.

### ***Final Thoughts***

Given the discussion of all the health benefits of blocking myostatin action, the value of the protein in mammalian evolution comes under question. Anecdotal evidence points to the temperament of double muscled animals as slower and more lethargic. Investigations on the endurance of double muscled animals has been contradictory (Qiao et al., 2008; Tang et al., 2007). Evolutionarily, the loss of control over muscle growth could have been a detriment to survival – double muscling in the wild could translate as a larger, slower prey. Indeed, the non-transgenic double muscled animals (cattle, sheep, whippet) were the result of artificial, not natural, selection. Myostatin null mice also eat more than wild type mice (Yang and Zhao, 2006). Thus, the caloric requirements for maintaining hypermuscularity could be relatively disadvantageous for some vertebrates. Future studies are needed, however, to determine why nullifying mutations have not arose throughout vertebrate evolution.

Myostatin blockade is potentially a great leap forward for the treatment of a variety of diseases. The number of people who possibly could be helped by myostatin blockade treatment

for heart disease, muscular dystrophy, and diabetes far outweighs the possible abuses. However, it is critical that guidelines for ethical use are followed as the technologies become available.

## CHAPTER FOUR

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