Identification of signaling cascades involved in myostatin-mediated repression of skeletal muscle growth

by

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ABSTRACT

Myostatin, a member of the TGF-β superfamily, negatively regulates skeletal muscle growth. Lack of functional myostatin is responsible for the double-muscled phenotype in cattle, which is characterized by an approximate 20% increase in muscle mass. Myostatin-knockout mice have individual muscles that weigh 2-3 times those of wild-type mice. In addition, an instance of human muscle hypertrophy resulting from a mutation in the myostatin gene has been reported. Despite recent advances in the identification of myostatin pathway components, including a putative receptor, the precise mechanism by which myostatin signals remains unknown. The objective of this study was to identify differences in gene expression between myostatin-null and wild-type mice at 3 developmental time points: 13.5 d.p.c., 17.5 d.p.c., and 35 days of age. With a false discovery rate (FDR) of 0.05, there were 472 genes differentially expressed at 13.5 d.p.c., 73 at 17.5 d.p.c., and 2109 at 35 days of age. At all three time points, changes were observed in genes involved in metabolism, enzyme activity, cell communication, and stimulus response. Further analysis focused on the results obtained at 35 days of age. At this time point, numerous changes were observed in genes implicated in translation and protein modification, differentiation, homeostasis, and energy production. In addition, the results indicated a switch in myogenic fiber type, with several genes encoding slow contractile protein isoforms down-regulated in myostatin-null tissue. It was also observed that a number of genes for Wnt pathway components, including Wnt4, were differentially expressed. Wnt4 has been shown to stimulate proliferation of a variety of cell types. Since it can also induce expression of myogenic regulatory

genes, and was upregulated in the absence of myostatin, it was hypothesized that Wnt4 may be able to promote proliferation of muscle satellite cells. In this study, exposure to Wnt4 stimulated proliferation of satellite cells obtained from wild-type mice. In contrast, the Wnt inhibitors sFRP1 and -2 decreased proliferation of myostatin-null satellite cells. Taken together, this research provides evidence of a role for Wnt4 in postnatal skeletal muscle growth and hypertrophy and offers insight into possible downstream effectors of myostatin signaling.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

Therapies aimed at increasing skeletal muscle mass have the potential to profoundly impact human health and quality of life. A number of conditions can result in muscle atrophy, including aging, chronic disuse, and exposure to microgravity environment, as well as diseases such as muscular dystrophies and AIDS. The ability to increase lean muscle mass is also of great benefit to overall fitness. In addition, the possibility of genetically or pharmacologically manipulating muscle growth in livestock species is highly desirable from an agricultural industry standpoint.

Research on the molecular regulation of muscle hypertrophy provides insight into potential targets whose manipulation may impact muscle growth. A number of growth factors and signaling pathways have been implicated in skeletal muscle hypertrophy. Of particular interest is myostatin, a TGF-β superfamily member that acts as a negative regulator of skeletal muscle growth. Lack of functional myostatin has been shown to cause muscle hypertrophy in cattle, mice, and, recently, a human. However, the details of the signaling pathway through which myostatin represses muscle growth remain unclear.

This study sought to identify potential downstream signaling targets of myostatin by analyzing differences in gene expression between myostatin-null and wild-type mice. The results suggest that myostatin influences genes involved in a remarkable range of cellular and biological processes, both during embryonic

development and postnatally. New insights are offered into factors which may interact with or be regulated by myostatin in the control of skeletal muscle growth. The exploitation of this information may prove useful in the identification of therapeutic targets for the treatment or prevention of muscle atrophy.

Thesis organization

This thesis is a discussion of the author's research presented in the alternative format. The first chapter contains a general introduction and a review of the relevant literature. Chapter 2 is a full-length manuscript to be submitted to the FASEB Journal and published online. The third chapter is a summary of the paper in Chapter 2 and is intended for publication in the print version of the journal. Chapter 4 consists of general conclusions regarding the research discussed herein. In Chapters 2 and 3, the contributions of the authors were as follows: C.A. Steelman was the primary researcher and author, under the direction of J.M. Reecy. All statistical analyses were completed by J.C. Recknor and D. Nettleton.

Review of Relevant Literature

Muscle Development

I. Overview

During embryonic development, the mesodermal precursor cells that will eventually become muscle arise from the dermomyotome portion of the somites. Specification and differentiation of myoblasts requires several myogenic regulatory factors (MRFs): MyoD, Myf5, myogenin, and MRF4. In addition, myogenesis is dependent on the complex interplay between a number of other signaling molecules

and transcription factors, including Sonic hedgehog (Shh), Wnts, Pax3, bone morphogenic proteins (BMPs), noggin, Notch, fibroblast growth factors (FGFs), Lbx1, Mef2, Msx1, and hepatocyte growth factor (HGF)/Scatter factor and its receptor, c-Met (reviewed in 1, 2).

Mononucleated myoblasts eventually cease proliferation and begin to terminally differentiate. First, they undergo cell cycle withdrawal. As differentiation progresses, they align, fuse together to form multinucleated myotubes, and begin to express a host of muscle-specific genes (reviewed in 3, 4). Embryonic myogenesis occurs in two major waves. The earliest myoblasts to develop are involved in primary myotube formation, while a later myoblast population undergoes fusion during secondary myogenesis. The fibers that arise from these two populations are morphologically distinct, with the primary fibers acting as a scaffold around which the secondary fibers develop (5). In the mouse embryo, primary myogenesis occurs around 11-13 days post-coitus (d.p.c.), with secondary myogenesis following at least two days later, at approximately 15-17 d.p.c. (6).

II. Role of the myogenic regulatory factors

A group of transcription-factor encoding regulatory genes (the MRFs) is specific to skeletal muscle. Each of these genes was identified based on its ability to induce conversion of non-muscle cells to the myogenic lineage, beginning with the identification of MyoD in 1987 (7). This was followed by the discovery of genes encoding three additional structurally homologous proteins, myogenin (8), Myf5 (9), and MRF4 (10). All four of the MRFs are basic helix-loop-helix (bHLH) proteins that form dimers with members of the E-protein family of transcription factors (11) and

bind to E-box elements (CANNTG) on target genes to modulate transcription (12, 13). In addition to the MRFs themselves, many other muscle-specific genes contain E-boxes, including desmin (14), muscle creatine kinase (15), myocyte enhancer factor-2 (Mef2) (16), and myostatin (17).

The functions of the MRFs in the regulation of muscle development have mainly been ascertained through the use of knockout mouse models. MyoD-null mice have normal muscle development, likely due to compensation by elevated Myf5 expression (18). When Myf5 is inactivated, early somite development is hindered, although the skeletal musculature appears normal. These mice die as neonates because of a major rib defect that impedes breathing (19). Interestingly, Myf5/MyoD double mutants exhibit a total lack of muscle formation and do not even have myoblasts (20). In myogenin knockouts, unfused mononucleated cells largely substitute for myofibers, and death occurs perinatally (21, 22). In mice that lack MRF4, skeletal muscle appears normal, possibly due to a substantial increase in myogenin expression. These mice also display rib abnormalities (23). These studies, taken in combination with the phenotypes of multi-MRF knockout mice and the expression patterns of the MRFs both in cell culture models and *in vivo*, reveal roles for MyoD and Myf5 in myogenic specification early in development, while myogenin and MRF4 are required for differentiation and later myogenesis. Although the knockout mouse phenotypes indicate that there may be some functional redundancy between the MRFs, it has been demonstrated that these factors do indeed have distinct roles for which the others are unable to compensate (24, 25).

Muscle Hypertrophy

I. Overview

Myofibers respond to environmental cues by shifts in the balance between protein synthesis and degradation. Atrophy, or loss of muscle mass, results when degradation outweighs myoblast proliferation and/or the accretion of new protein. Hypertrophy, on the other hand, is caused by the opposite scenario. This results in augmented fiber size without an increase in fiber number (hyperplasia). In mammals, the number of muscle fibers is typically established during fetal development, with the vast majority of postnatal muscle growth attributable to fiber hypertrophy (26). The muscle growth that accompanies exercise is also due to fiber hypertrophy.

Different signaling molecules and pathways have been implicated in muscle hypertrophy. Insulin and insulin-like growth factor (IGF)-1 modulate the hypertrophic response through a mechanism involving phosphoinositol 3-kinase (PI3K) and Akt1 (27). IGF-1 is capable of inducing an increase in fiber size both in cultured myotubes (28) and when overexpressed in mice (29, 30). In addition, IGF-1 expression is upregulated in humans and rats during overload-induced hypertrophy (31, 32). Other signaling molecules that may play a role in fiber hypertrophy include fibroblast growth factors (FGFs) (33), leukemia inhibitory factor (LIF) (34), hepatocyte growth factor (HGF) (35), calcineurin (36), and myostatin (37).

Animal models of muscle hypertrophy have been studied to determine the genetic factors that may contribute to increased muscle mass. Naturally occurring mutations lead to the callipyge phenotype in sheep (38) and the double-muscled

condition in cattle (39). Methods for inducing skeletal muscle hypertrophy in animals in a laboratory setting include functional overload by removal or damage of a synergistic muscle, resistance training, and chronic stretch (reviewed in 40).

II. Role of satellite cells

Muscle growth that occurs postnatally or as a result of mechanical loading is largely attributable to satellite cells. Satellite cells are quiescent, mononucleated cells that are located at the periphery of the muscle fiber, above the basal lamina but under the sarcolemma (41). They arise later in embryonic development, are believed to be distinct from other myoblast populations (reviewed in 3, 5), and can contribute to postnatal muscle growth (42). Satellite cells were traditionally identified by electron microscopy based on their characteristic location and their high nuclear-to-cytoplasmic ratio (41). Other characteristics of quiescent satellite cells include smaller nuclei with higher levels of densely packed heterochromatin, and fewer organelles (3, 44). It is thought that *Pax7* is required for their specification, since *Pax7*-null mice reportedly lack satellite cells (45). Interestingly, these mice can still undergo postnatal growth, although attenuated (46). It has recently been reported that *Pax7*-null mice do, in fact, have satellite cells, although the pool is diminished and the numbers fall off rapidly after birth (47).

Satellite cells can emerge from their quiescent state and become activated to proliferate and fuse with each other or with existing muscle fibers to allow for muscle growth or repair (48, 49). Several circumstances bring about satellite cell activation, including growth (42), injury (49), ablation of synergistic muscles (50-52), denervation (53), and exercise-induced overload (54). In humans, strength training

has been shown to cause satellite cell activation (55, 56). Once activated, satellite cells begin to express myogenic genes, including *Myf5* and *MyoD* (57). It is believed that hepatocyte growth factor (HGF)/scatter factor is the mitogen responsible for satellite cell activation (35, 58). A number of other cytokines have been shown to stimulate satellite cell proliferation or differentiation, including insulin-like growth factor (IGF)-1 (59), fibroblast growth factors (FGFs) (60), leukemia inhibitory factor (LIF) (61), and interleukin (IL)-4 (62). Maintenance of the satellite cell population results when a portion of the activated cells exit the cell cycle prior to differentiation and return to a mitotically quiescent state. It is believed that Pax7 plays a role in this self-renewal process (63, 64).

Much research has been undertaken in a quest to identify specific markers that can be exploited to distinguish satellite cells from other cell types. Either quiescent or activated satellite cells can be identified based on expression of CD34, Myf5 (65), c-Met, M-cadherin (66), Pax7 (45), myocyte nuclear factor (MNF) (67), and Syndecan-3 and -4 (68, 69).

Satellite cell density is age- and fiber-type-specific. In neonatal mice, satellite cells compose about 30% of the nuclei in muscle, but this number tapers off drastically, to less than 5%, in the first two months of life (70). Aged muscle has an impaired ability to recover from atrophy (71), which is likely due to depletion of satellite cells and a decrease in their ability to proliferate (72-74). Several studies have cited higher numbers of satellite cells in primarily slow versus fast muscles (reviewed in 3). Even in mixed fiber-type muscles, there seem to be differences in the percentage of satellite cells present on individual slow and fast fibers (75).

III. Identification of satellite cells--Myf5nlacZ mouse

Mice in which a transgene for nuclear-localized β-Galactosidase (*nlacZ)* has been targeted to the *Myf5* locus provide a useful tool for the identification of myogenic cells (76, 77). The *nlacZ* allele is expressed early in embryonic development (8.5 d.p.c.), from the beginning of myotome formation. At birth, its presence is detected in all skeletal muscle, reportedly in all nuclei therein (78). In the adult animal, its expression is limited to satellite cells. However, it has been found that a small population of quiescent satellite cells is not positive for β-Galactosidase (65). In regenerating muscle, two classes of activated satellite cells have been detected: those that express both β-Gal and MyoD, and those that express MyoD only (57). This demonstrates that all of the β-Gal positive cells are myogenic. These studies indicate that staining for β-Galactosidase reliably identifies satellite cells from the adult Myf5nlacZ mouse, although a small percentage may be ignored as a result.

TGF-β Superfamily

I. Overview

The many members of the transforming growth factor (TGF)-β superfamily of cytokines are involved in a wide variety of cellular processes, including specification, recognition, migration, proliferation, differentiation, and apoptosis. They exert their effects on virtually all cell types and during all stages of development in organisms from *Drosophila* to mammals. Subfamilies that are part of the superfamily include TGF-βs, activins, bone morphogenic proteins (BMPs), and growth and differentiation

factors (GDFs). Typically, TGF-βs and activins are grouped together based on sequence homology and mechanism of signal transduction, as are BMPs and GDFs. Family members act as both activators and repressors of target gene expression. The transcriptional response elicited upon signaling depends not only on the ligand and receptor, but on the cell type and environment.

Members of the superfamily have a number of structural and functional characteristics in common. All contain a conserved pattern of cysteine residues that form a so-called "cysteine knot" structure (79). Their prepropeptides undergo proteolytic processing, and the active portion functions as a dimer, usually connected by a disulfide bond. Both monomers are able to bind two types of receptor, known as type I and type II (reviewed in 80). The TGF-β dimer is secreted in a latent complex, in which it interacts non-covalently with its own propeptide, the latency-associated peptide (LAP). Latent TGF-β-binding protein (LTBP) is also a component of this complex. In order for TGF-β to be functional, the complex must be dissociated (81, 82).

TGF-β ligands signal through a group of receptor serine/threonine kinases. Ligand binding at the cell surface causes formation of a heteromeric complex of a type I with a type II receptor. The type II receptor kinase domain then phosphorylates the type I receptor, which in turn signals by phosphorylating and activating the SMAD proteins, which function as transcriptional mediators (reviewed in 80). In humans, there are seven type I and five type II receptors that are involved in TGF-β signaling (83). TGF-βs and activins bind the extracellular domain of a type

II receptor, which recruits the type I receptor. They are unable to bind type I receptors directly. BMPs, on the other hand, seem to require both types of receptor and bind cooperatively (reviewed in 84).

Eight SMAD (SMA/MAD-related) proteins, SMAD1-8, are found in vertebrates. They were identified as homologues of *Mad* in *Drosophila* and the *sma* genes in *C. elegans*. The SMADs can be divided into three classes: receptor SMADs (1, 2, 3, 5, and 8), co-SMADs (4), and inhibitory or antagonistic SMADs (6 and 7). The receptor (R-) SMADS are serine-phosphorylated by the type I receptors. SMADs 2 and 3 propagate TGF-β and activin signals, while SMADs 1, 5, and 8 function in BMP signal transduction. Upon phosphorylation, R-SMADs complex with the common co-SMAD, SMAD4. This complex then translocates into the nucleus to mediate target gene expression. Transcriptional control is achieved both through binding to five-base-pair SMAD binding elements (SBEs) in the DNA, and by association of the SMAD complex with coactivators or corepressors, which can bind to their own target sequences. Since binding of SMADs to SBEs is both weak and non-specific for individual SMAD proteins, it seems that other DNA-binding cofactors are required to elicit the effects on gene expression in a particular cell type. The I-SMADs can inhibit signaling by preventing association of R-SMADs with type I receptors, and possibly by other methods as well. SMAD7 antagonizes both TGF-β and BMP signaling, while SMAD6 seems to act specifically against BMPs. It appears that the I-SMADs act through a negative feedback mechanism, as expression of Smad7 is induced by TGF-β (80, 84, 85, reviewed in 86). It seems

likely that ubiquitin-mediated degradation of the SMAD proteins is a critical mechanism for the tight control of TGF-β signaling (87-89).

In some cases, TGF-β signal transduction has been shown to occur through different pathways, which may or may not result in phosphorylation of the SMAD proteins. In *Xenopus,* the Ras/MAP kinase pathway has been demonstrated to affect SMAD1 function in neural development (90). Other MAPK pathway components, including JNK and p38, may be potential targets of TGF-β (reviewed in 88, reviewed in 91).

The role of TGF-βs in a wide variety of tissue types and developmental stages from embryo to adult have been extensively studied. A multitude of factors interact to control signaling at all levels. Members of the superfamily function in processes ranging from patterning of the embryo and organogenesis, to hematopoiesis, wound healing, and regulation of the immune response. Of note is the tendency of TGF-β to inhibit the proliferation of numerous cell types. This occurs by prevention of cell cycle progression from G_1 phase, either via repression of cyclin-dependent kinases (CDKs) or c-Myc. In other cases, TGF-β signaling leads to induction of apoptosis. Given TGF-β's ability to affect both cell growth and death, it is logical that mutations in signaling pathway components, including receptors and SMADs, can lead to oncogenesis. These mutations have been identified in large percentages of ovarian, colon, and pancreatic cancers. Paradoxically, TGF-β can either act as a tumor suppressor or promote metastasis, depending on the stage of the tumor (reviewed in 84, 92).

II. TGF-β signaling and skeletal muscle

A number of studies have focused on the roles of TGF-β in the formation of muscle, as well as its effects on cultured myoblasts. Treatment of embryonic stem cells with TGF-β induces entry into the myogenic lineage (93). In cooperation with other signaling molecules, TGF-β1 functions to stimulate myogenesis in explanted somites (94). In mesangioblasts, stem cells capable of forming mesodermal derivatives, incubation with TGF-β results in smooth muscle differentiation (95). Addition of relatively low (pM) concentrations of TGF-β to muscle cells under low-serum conditions strongly inhibits differentiation, including expression of muscle-specific genes, but does not seem to hinder the cells' ability to proliferate (96, 97). Specifically, treatment with TGF-β1 has been shown to negatively regulate expression of both *MyoD* (98) and *myogenin* (99). Incubation of cultured satellite cells or rat neonatal myoblasts with TGF-β results in a dose-dependent decrease in both cell proliferation and differentiation (100). Interestingly, under growth conditions (in the presence of mitogens) addition of TGF-β to culture media can enhance myoblast differentiation (101). Inhibition of differentiation seems be mediated, at least in part, by SMAD3. SMAD3 can associate with MyoD and prevent it from dimerizing with E-proteins, which means it can no longer bind to E-boxes in the DNA and activate target gene expression (102). It has recently been shown that SMAD3 can also repress the activity of MEF2, which is required as a cofactor in MRF-mediated transcription (103). Differentiation inhibition by TGF-β may be due in part to hindrance of Ca^{2+} signaling, which is required for fusion. In mouse primary cells,

TGF-β treatment causes a drop in the number of functional calcium channels, as well as their conductance (104). Direct injection of recombinant TGF-β1 into mouse muscle induces autocrine production of TGF-β1, which results in fibrosis and the development of scar tissue (105).

Activin has also been shown to impact muscle development. Activins are expressed at sites of muscle development during embryogenesis (106-108) and function to inhibit myoblast proliferation and differentiation. This inhibition has been attributed to repression of MyoD, along with either Myf5 (108) or Pax3 (109).

Myostatin

I. Discovery

Myostatin, also known as growth and differentiation factor (GDF)-8, is a fairly recently discovered member of the TGF-β superfamily, having been identified in a screen for new members in 1997 (37). It possesses the major trademarks of the superfamily, including the requisite nine cysteine residues in its C-terminus and its requirement for proteolytic processing of the prepropeptide to form a functional protein. The myostatin (*Mstn)* gene consists of three exons and two introns and yields a 2.9 kb mRNA (37, 110). It is initially translated as a 376-amino-acid (52-kDa) product, which undergoes two cleavage events to yield 27-kDa N-terminal and 12.5-kDa C-terminal fragments. The N-terminus contains a sequence which signals for secretion, and the functional protein is a disulfide-linked dimer of the C-terminal portion. During polyacrylamide gel electrophoresis under non-denaturing conditions, 101-kDa (unprocessed) and 25-kDa (processed) protein species are detected (37).

II. Expression

Myostatin is expressed almost exclusively in skeletal muscle. Its expression in the mouse embryo is first detected in the myotomal compartment of the anterior developing somites at 9.5 d.p.c. It continues to be expressed throughout development and into adulthood, although its expression level varies between muscles (37). In the bovine embryo, myostatin transcript has been detected at all timepoints examined, beginning at day 15. Expression increases dramatically at day 31 and is elevated throughout gestation (111). Myostatin has been detected at lower levels in adipose (37), mammary (112), and cardiac (113) tissues in mammals, and in the brain of both brook trout and yellow perch (114).

III. Gene disruption

Targeted disruption of the myostatin gene in mice by deletion of the portion encoding the C-terminus results in a substantial increase in muscle mass. Animals homozygous for the mutant allele have individual muscles that weigh 2-3 times those of their wild-type counterparts, while heterozygotes display an intermediate phenotype. This hypermuscularity has been attributed to a combination of both hypertrophy and hyperplasia (37). Myostatin-null mice also exhibit significantly lowered fat deposition as compared to wild-type controls (115, 116). In mouse models of obesity, lack of myostatin has been shown not only to decrease fat accumulation, but to positively affect glucose metabolism, resulting in mice that are less prone to diabetes (115).

Interestingly, the manifestation of the mutant phenotype seems to depend on the nature of the mutation. In transgenic mice expressing a dominant-negative

myostatin which has an altered cleavage site, the observed increase in muscle mass is due to hypertrophy, not hyperplasia (117). On the other hand, hyperplasia without hypertrophy is present in mice with a missense mutation that causes substitution of a tyrosine for one of the cysteines in the C-terminus (118). In contrast, male transgenic mice expressing myostatin under control of the muscle creatine kinase promoter, resulting in muscle-specific overexpression, have reduced muscle mass, including decreased fiber cross-sectional area and number of myonuclei (119). It has also been determined that the "compact" (*Cmpt)* phenotype, from a line in which mice were selected for high carcass protein yield, is caused by a mutation in the propeptide-encoding region of the myostatin gene (120). In fact, introduction of this particular mutation into an inbred high-growth line resulted in a significant increase in muscle weights, accompanied by a reduction in fat deposition (121).

A similar hypermuscular, or "double-muscled," phenotype was described in cattle in the 1800s (39). In breeds such as the Belgian Blue and Piedmontese, two of the most commonly studied double-muscled breeds, the phenotype is characterized by an approximate 20% increase in muscle mass (122). Research indicated that the phenotype was likely caused by a single gene on bovine chromosome 2, which was coined the *mh,* or muscle hypertrophy, locus (123). The increased muscularity observed in cattle has been attributed primarily to hyperplasia (124, 125). The musculature of these animals is emphasized by their leanness, and their intramuscular fat stores are especially diminished (126). Although double-muscled cattle may be desirable in terms of meat production, they present several management issues. Potentially troublesome traits include reduced stress

tolerance, decreased bone mass, hypotrophy of internal organs, reduced female fertility, and difficulty calving (dystocia) (125, 127-130). To alleviate the reproductive problems associated with double-muscled cows, one group has proposed generating lines in which only the males are hypertrophic. They have recently created transgenic mouse lines in which the males have 5-20% more skeletal muscle than controls (131).

Based on the similarities between the phenotypes of myostatin-null mice and double-muscled cattle, and the fact that the myostatin gene is highly conserved across species (132), it was reasonably determined that this gene was a strong candidate for causation of the heavy musculature in cattle. Indeed, it was discovered that double-muscled Belgian Blue cattle are homozygous for an 11 bp deletion, which results in a frame shift (111, 132, 133), while Piedmontese animals have a missense mutation that causes a conserved cysteine residue to be replaced by a tyrosine (111, 132). The latter has been reported to cause disruptions in myostatin processing, as the affected amino acid is required for correct folding into the "cysteine knot" structure (134).

Recently, the first instance of a myostatin mutation resulting in muscle hypertrophy in a human was reported (135). A German child was noted, from birth, to exhibit remarkable musculature. He was later found to have a mutation in the myostatin gene that results in missplicing of the transcript, therefore a lack of functional protein. This cements the belief that the ability to manipulate myostatin expression may provide a means to enhance muscle development in patients who are experiencing atrophy.

IV. Postnatal manipulation

Evidence indicates that myostatin can also exert its effects on muscle when its expression is manipulated after birth. When myostatin is inactivated postnatally in skeletal muscle, using the cre/lox system, mice exhibit enhanced musculature, due mostly to hypertrophy (136). Adult mice in which myostatin is inhibited by antibody treatment also show an increase in skeletal muscle mass, attributed to hypertrophy, as well as improved grip strength (137). In contrast, mice in which myostatin is overexpressed by injection of myostatin-producing CHO cells display severe wasting of skeletal muscle and adipose tissue (138).

V. Myostatin and fiber type

Several studies have noted an association between myostatin expression and fiber type. Carlson *et al.* (139) identified a propensity for myostatin to be expressed in fast-twitch glycolytic fibers of the mouse hindlimb, based on a strong correlation between the expression of myostatin and myosin heavy chain (MHC) type IIb. In addition, myostatin promoter constructs injected into mouse quadriceps have been found to activate reporter gene expression specifically in fast fibers (140). In myostatin-null mice, both fast- and slow-twitch muscles exhibit an elevated percentage of fast glycolytic fibers and a corresponding decrease in slow fiber type (141). Studies comparing normal to double-muscled cattle support these findings, as double-muscled animals have an increased proportion of fast-twitch fibers both during gestation (142) and at 18-22 months of age (143).

VI. Regulation and activation

Myostatin has been shown to circulate in a latent complex in the serum of mice (138) and humans (144). Like TGF-β, which exists in an inactive form bound to its propeptide and latent TGF-β binding protein (LTBP) (81, 145, 146), the multi-component myostatin complex must be dissociated for the protein to be functional. Myostatin has been demonstrated to noncovalently associate with and be inhibited by its propeptide both *in vitro* (138, 147) and *in vivo* (148, 149). Transgenic mice overexpressing the propeptide exhibit hypermuscularity comparable to that observed in myostatin knockouts (149, 150). Promoter induction by myostatin in cultured rhabdomyosarcoma cells is hindered by incubation with propeptide (151).

Serum myostatin has been shown to be bound not only to its propeptide, but to a protein with significant homology to follistatin, follistatin-related gene (FLRG), which can inhibit myostatin activity in cultured cells (148). Follistatin is known to bind to and antagonize activin (152, 153), but the range of defects observed in follistatin-null mice indicates that it may regulate other growth factors as well (154). In fact, the ability of recombinant follistatin to hinder myostatin activity in reporter assays (138, 151) and the highly muscled phenotype observed in mice with muscle-specific overexpression of follistatin (149) provide support for the role of follistatin as a myostatin antagonist. Two-hybrid analysis has been used to demonstrate that these two proteins interact directly with one another (155).

Another protein with some similarity to follistatin has also been demonstrated to bind to and inhibit myostatin. This protein, growth and differentiation factor-associated serum protein-1 (GASP-1), is also capable of binding myostatin's

propeptide. Affinity purification and mass spectrometry indicate that it associates with circulating serum myostatin (156). Based on these studies, it is believed that the latent complex consists of the myostatin C-terminal dimer, its propeptide, and FLRG or GASP-1, or both. Precisely how these components are associated with each other is unknown.

It is uncertain how myostatin activation occurs *in vivo*. As has been shown for TGF-β (157), acid treatment leads to dissociation of the myostatin latent complex and release of active myostatin *in vitro* (138). It is possible that activation *in vivo* may be accomplished by cleavage of the propeptide by metalloproteases. When membrane-bound and matrix metalloproteases of the metzincin family are inhibited in C2C12 myoblasts, the cells' fusion is enhanced and they undergo myotube hypertrophy. Unprocessed, full-length myostatin is also present only in treated cells, indicating that the observed hypertrophy may be due to myostatin's failure to be activated (158). Furthermore, incubation of purified latent complex with several members of the bone morphogenetic protein-1/tolloid (BMP-1/TLD) metalloproteinase family results in cleavage products visible by western blot, and reporter assays indicate that myostatin is rendered functional following cleavage. In addition, when propeptide with a mutated cleavage site is injected into mice, a significant increase in muscle mass is observed, indicating that these enzymes may be required for *in vivo* activation of the myostatin latent complex (159).

VII. Purported signaling mechanisms

While the precise mechanism through which myostatin signals to inhibit muscle growth is as yet unknown, evidence suggests that it is capable of signaling in

a TGF-β-like manner. A myostatin-specific receptor has not been discovered. Instead, it appears likely that myostatin functions through activin receptor type IIB (ActRIIB). Cross-linking studies show that myostatin can bind to both ActRIIB, and, with lesser affinity, to ActRIIA (149, 160). Transgenic mice expressing a skeletal-muscle-specific, dominant-negative form of ActRIIB display a musclular phenotype similar to that of the myostatin-null mouse, as a result of both hyperplasia and hypertrophy (149). However, it is impossible to attribute the phenotype entirely to myostatin's inability to initiate signaling, since various other ligands also bind to this receptor, including activins (161-163), BMPs (164, 165), GDF-5 (166), and nodal (167). The type II activin receptors can interact with a variety of type I receptors, depending on which ligand propagates the signal (84). In cell culture, myostatin-receptor complexes were only formed when ActRIIB was coexpressed with ALK4 (ActRIB) or ALK5 (TβRI). As is the case for TGF-β (84), myostatin treatment of cultured cells results in phosphorylation and activation of SMADs 2 and 3 (151, 160, 168). A recent study concluded that myostatin signaling via SMADs 2 and 3 is negatively regulated by inhibitory SMAD7, because the ability of recombinant myostatin to stimulate transcription of a TGF-β-responsive reporter in rhabdomyosarcoma cells is drastically reduced when the reporter is cotransfected with SMAD7 (151). SMAD7 also inhibits TGF-β signal transduction (169, 170). Myostatin treatment is able to stimulate SMAD7 promoter activity, indicating that myostatin and SMAD7 may regulate one another's activity through a negative feedback loop (151).

Recent research indicates that myostatin may also be able to exert its effects on skeletal muscle through a pathway independent of the SMADs. Immunoblots from myostatin-treated cultured cells have shown activation of p38 MAP kinase, as well as SMAD2. It has been determined that p38 phosphorylation occurrs via a MAPKKK, TGF-β-activated kinase-1 (TAK1), through a SMAD-independent mechanism (171). Other TGF-β superfamily members have also been shown to signal through TAK1, triggering the p38 MAPK pathway (172-174).

VIII. Effects on cell proliferation and differentiation

Several studies have demonstrated that myostatin inhibits cell proliferation. The addition of myostatin to cultured C2C12 cells reportedly causes a dose-dependent decrease in proliferation (175, 176). At lower concentrations, it has been shown to hinder proliferation of myogenic cells from porcine embryos (177). Transient transfection of C2C12s with a myostatin construct also results in inhibition of proliferation (178). In cultured C2C12 cells, the addition of recombinant myostatin has negative effects on DNA and protein synthesis, although the effect is more pronounced in myotubes than myoblasts (175). C2C12s stably overexpressing myostatin display impaired proliferation and decreased apoptosis compared to cells expressing an antisense construct (179).

Myostatin seems to exert its effects on proliferation by regulating cell cycle progression. Cell cycle arrest is heralded by activation of p21, which inhibits several cyclin-dependent kinases, or Cdks, including Cdk2. Without active cyclin-CDK complexes to phosphorylate and inactivate retinoblastoma protein (Rb), the cell cycle cannot progress. Based on its ability to induce an increase in levels of p21

and apparent repression of Cdk2, as well as the presence of retinoblastoma protein in a largely hypophosphorylated form, it is hypothesized that myostatin causes cell cycle arrest by inhibiting the G_1- to S-phase transition (176, 178-180). In RD cells, a rhabdomyosarcoma tumor cell line derived from skeletal muscle progenitors that fail to differentiate, treatment with recombinant myostatin has been shown to block proliferation, but p21 expression and Rb phosphorylation status are unchanged (181). This suggests the availability of an alternative mechanism by which myostatin can inhibit cell proliferation.

It has also been suggested that myostatin negatively regulates myoblast differentiation. The addition of recombinant myostatin to cultured myoblasts has been shown to decrease the mRNA and protein levels of MyoD and myogenin (168). However, experiments using stably transfected, myostatin-expressing cell lines have had varying results. Ríos *et al.* reported that myostatin overexpression resulted in diminished transcript amounts for MyoD, myogenin, and creatine kinase, an indicator of terminal differentiation (182). Joulia *et al.*, on the other hand, saw no significant effect on myogenin message and inconsistent results when it came to MyoD mRNA. Yet, their data did indicate downregulation of both MyoD and myogenin protein levels (179). When cultured chicken fetal myoblasts were treated with recombinant myostatin and changes in gene expression were analyzed, several markers of differentiated muscle were shown to be down-regulated, including muscle creatine kinase, myosin regulatory light chain, and troponin C (183).

Since myostatin seems to be capable of regulating muscle fiber size, it would be reasonable to conclude that it could potentially regulate the proliferation

and fusion of satellite cells. Recently, it has been demonstrated that myostatin inhibits activation of satellite cells from quiescence and impairs their ability to self-renew (180). Several lines of evidence support this conclusion. First of all, when 4-week-, 8-week-, and 6-month-old mice were injected with BrdU prior to isolation of satellite cells from the tibialis anterior muscle, a significantly higher percentage of activated (proliferating) cells was present in myostatin-null than in wild-type muscle at all three ages. Myoblasts derived from satellite cells isolated from myostatin-null muscle also had a much higher proliferation rate than those from wild-type, irrespective of whether the muscle consisted primarily of fast- or slow-twitch fibers. This difference was nullified when 3.5-4.0 μg/ml recombinant myostatin was added to myostatin-null cultures (180). Another study showed that satellite cells isolated from older (6 or 7 months) myostatin-null mice exhibit increased rates of both proliferation and differentiation compared to those from wild-type mice (184). Flow cytometry analysis of satellite cells revealed a two-fold increase in the number of cells in S-phase in myostatin-null over wild-type mice. After these cells had been synchronized to G_1, wild-type cells exhibited slower progression into DNA synthesis, leading the authors to conclude that the presence of myostatin interferes with S-phase entry (180).

A recent study calls into question the use of recombinant myostatin in cell culture experiments. Ríos *et al.* (185) tested the effects of endogenous versus exogenous myostatin on cultured muscle cells. They determined that endogenous myostatin, which was transfected into myoblasts, thus overexpressed, was capable of activating a TGF-β-responsive promoter. Conversely, recombinant myostatin,

either produced in an insect cell line or commercially available, failed to induce reporter gene transcription in cultured C2C12 or L6 myoblasts, unless its concentration was elevated to 1 μg/ml. Studies cited above, in which recombinant myostatin was determined to inhibit proliferation or differentiation of myoblasts, utilized concentrations of greater than 2 μg/ml (168, 175, 176). TGF-β, on the other hand, managed to induce reporter expression at a concentration of only 1 ng/ml. This indicates that the high concentrations required to exert an effect on cultured muscle cells may be well above physiological levels. Interestingly, the levels of recombinant myostatin that had no effect on myoblasts were able to elicit reporter activity in hepatic and mammary carcinoma cell lines (185). It is possible that the reduced activity of recombinant myostatin, especially that which is produced in bacteria, is due to incomplete processing and folding. This issue has recently been addressed by Jin *et al.* (186).

IX. Myostatin and skeletal muscle disease

Myostatin expression has been examined in a variety of disease states in skeletal muscle. Of major interest is the role of myostatin in muscular dystrophies. In the *mdx* mouse, a model for Duchenne muscular dystrophy (DMD), loss of myostatin has been shown to improve the dystrophic phenotype (187, 188). DMD results from a mutation in the dystrophin gene, leading to lack of functional protein. The diaphragm is the most severely affected muscle in the *mdx* mouse, and most closely mimics the human phenotype. Repeated cycles of degeneration and regeneration result in muscle fibrosis and replacement of muscle with fatty tissue (189, 190). Improvement of the phenotype is observed when myostatin-null mice

are crossed with *mdx* mice. *Mstn*$^{-/-}$*/mdx* mice have more muscle mass and increased grip strength compared to *Mstn*$^{+/+}$*/mdx* counterparts (188). When *mdx* mice were injected weekly for three months with a myostatin-blocking antibody, the result was an increase in muscle size and weight, improved strength, and a reduction in degeneration (187). Similar positive effects on the dystrophic phenotype were observed when *mdx* mice were treated with recombinant myostatin propeptide. In this case, improvement in force generation was reported to be even greater than that seen after antibody treatment (191). These data suggest that myostatin-blocking therapies may have some clinical benefit when it comes to alleviating the symptoms of muscular dystrophy. However, elimination of myostatin in *dy* mice, which are models for laminin-deficient muscular dystrophy, does not decrease the severity of the phenotype, even though regeneration ability is improved (192).

Injection of toxins such as notexin or cardiotoxin into skeletal muscle causes necrosis and regeneration. Several studies have shown increased levels of either myostatin mRNA or protein following such injury (193-195), suggesting the possibility of a role for myostatin in the regeneration process.

It has been hypothesized that myostatin may contribute to muscle atrophy due to disuse. An increase in either mRNA or protein levels has been detected in patients following bed rest (196) or chronic disuse due to osteoarthritis (197). Hindlimb unloading is a common model for disuse atrophy in which mice or rats are suspended by the tail, so their hind feet are unable to touch the cage floor. Much research has been done using various combinations of unloading and reloading.

Wehling *et al.* (198) found an increase in plantaris myostatin mRNA and protein levels after ten days of unloading, but the amounts were no different from control animals after four days of reloading. Carlson *et al.* (139) saw a significant increase in myostatin mRNA after only one day of suspension, but only in the primarily fast-twitch gastrocnemius and plantaris muscles. The slow-twitch soleus exhibited significant atrophy, but had no detectable *mstn* transcript. Another group saw no significant differences in myostatin protein levels after up to 14 days of hindlimb unloading. However, a dramatic decrease was noted after two days of reloading (199). Since myostatin inhibits muscle growth, it was assumed that myostatin-null mice would undergo less atrophy than wild-type controls during hindlimb unloading. On the contrary, knockouts actually lost a higher percentage of their body weight and muscle mass than controls. Based on expression patterns of the MRFs, it appeared that the null mice failed to initiate regeneration to counter muscle loss during suspension (200).

Increased levels of myostatin are also observed in a number of other instances of muscle atrophy. Serum myostatin protein levels are higher in men with AIDS wasting disease than in both HIV-infected men who have not undergone significant muscle loss and healthy controls (144). One study noted an association between aging-related muscle atrophy and increased serum myostatin (201), although no differences were observed in a comparison of muscle myostatin mRNA levels between young and older men (202). Rats who have undergone muscle atrophy due to spaceflight show augmented expression of myostatin transcript and protein, although the levels return to normal after a period of time in normal gravity

(203). Also, when atrophy is incited by treatment with glucocorticoids, rats display elevated intramuscular myostatin levels (204). Similarly, rats in which muscle atrophy has been induced by denervation exhibit an initial spike in myostatin expression, although this is followed by a significant decline in mRNA and protein levels by two weeks after surgery (205). This research suggests an important role for myostatin signaling in the loss of muscle mass associated with a variety of circumstances.

Wnt Signaling

I. Overview

The Wnt signaling pathway plays a number of critical roles during embryonic development and functions in adult tissue homeostasis as well. Its members are typically highly conserved throughout both vertebrate and invertebrate species, and are involved in cell proliferation and polarity, patterning of the embryo, and cell fate decisions. Nineteen Wnt proteins exist in mammals, all of which are secreted glycoproteins containing a number of conserved cysteine residues (reviewed in 206). Palmitoylation of one of the cysteines is required for the protein to be functional (207). The response to Wnt signaling is cell-type and context specific. There are two principal pathways that can be activated by Wnts: the canonical pathway, through β-catenin (reviewed in 206, 208), and the Wnt/calcium pathway (reviewed in 209, 210). Another non-canonical pathway is one through which Wnt signals control specific cell movements during vertebrate gastrulation, which evidence indicates may be analogous to the planar cell polarity pathway during development in *Drosophila* (reviewed in 211).

Different intracellular effectors are involved in Wnt signal transduction via each of the pathways. Past research has indicated that the Frizzled receptors are the proteins primarily responsible for dictating the specific pathway through which signaling occurs (reviewed in 210). However, a recent study shows that the decision may be due to the ability of a particular Wnt to interact cooperatively with Frizzled and a co-receptor, LRP (212). It has been suggested that there may be multiple non-canonical Wnt pathways, although it remains to be seen if these pathways are distinct or interconnected. In *Xenopus* embryos and mammalian cells, Wnt signaling has been shown to activate Jun N-terminal kinase (JNK) and Rho-family GTPases (213). It may also be able to moderate cyclic GMP signaling (214). Much less is understood about non-canonical than canonical Wnt signaling, and the downstream targets remain controversial.

II. Canonical pathway

Signal transduction through the canonical Wnt cascade is governed by the phosphorylation status of β-catenin. When no ligand/receptor interaction occurs and the pathway is "off," β-catenin is targeted for proteosome-mediated degradation by associating with Axin and adenomatous polyposis coli (APC), which allows it to be phosphorylated by glycogen synthase kinase (GSK)3. In this case, without nuclear accumulation of β-catenin, target genes are not activated (reviewed in 206). Recent studies have demonstrated that β-catenin may alternatively undergo degradation by calpain (215, 216). When the signaling pathway is active, secreted Wnts interact with Frizzled seven-transmembrane receptors, in combination with low-density lipoprotein receptor-related protein (LRP), at the cell membrane. Following

ligand/receptor binding, the signal is transduced through Dishevelled (Dsh/Dvl), which may interact with Axin to cause Axin degradation. GSK3 is also inhibited, so β-catenin begins to accumulate and translocate into the nucleus. To activate target gene expression, it interacts with transcription factors of the lymphoid enhancer-binding protein (LEF) and T-cell factor (TCF) families. Primary inhibitors of Wnt signaling include Dickkopf (Dkk), which binds LRP and causes it to become sequestered inside the cell, and the secreted Frizzled-related proteins (sFRPs), extracellular proteins that bind Wnts (reviewed in 206).

III. Wnt/calcium pathway

Wnt signaling through the Wnt/calcium pathway acts independently of β-catenin. In this pathway, binding of Wnts to Frizzled receptors triggers an accumulation of intracellular Ca^{2+} ions. Heterotrimeric G-protein subunits are activated upon ligand-receptor binding and activate phospholipase C (PLC), which initiates a chain of events to allow Ca^{2+} release. Protein kinase C (PKC), Ca^{2+}/calmodulin-dependent protein kinase II (CamKII), and/or calcineurin are activated by intracellular Ca^{2+} (reviewed in 209). Dishevelled, localized at the plasma membrane, also seems to be involved in Wnt/Ca^{2+} signaling (217). Another downstream effector of this pathway may include the transcription factor nuclear factor of activated T-cells (NF-AT), a target of calcineurin (218).

IV. Wnt signaling and skeletal muscle

Wnt signaling has been demonstrated to play a role in myogenesis, during both embryonic development and postnatal muscle regeneration. Münsterberg and Lassar (219) concluded that signals from both the notochord/floor plate and the

neural tube are required for expression of myogenic regulatory genes and myogenesis in early somites. Based on *in vitro* explant experiments from chick embryos, they later determined that Sonic hedgehog (Shh) produced by the notochord/floor plate likely acts together with Wnt signals, specifically Wnts1, -3, and -4, from the dorsal neural tube to induce myotome development. Seemingly, Shh is necessary only during early stages of somite development (220). Fan *et al.* (221) co-cultured Wnt-expressing COS cells with explants of presomitic mesodermal tissue and determined that Wnts1, -3a, -4, and -6 are sufficient to induce and maintain dermomyotome formation, as evidenced by the expression of the dermomyotomal markers *Pax3*, *Sim1*, and *Pax7*. Their results indicate that Wnts1 and -3a are more likely to act as diffusible factors than Wnts4 and -6, which are more effective during cell-cell contact. The necessity of Wnt1 and Wnt3a expression in the dorsal neural tube is underscored by the discovery that double mutant embryos exhibit a lack of medial dermomyotome (222). It has also been shown that Wnt7a, which is expressed in the dorsal ectoderm, is sufficient to induce *MyoD* expression in mouse embryo explants. Wnt1, which is most highly expressed in the neural tube, preferentially stimulates induction of *Myf5.* Several Wnts, including Wnts4, -5a, and -6, are able to induce both of these MRFs (223). Interestingly, Wnt1 and Wnt7a likely exert their effects via different pathways, as Wnt1 is believed to signal through β-catenin (224) and Wnt7 by way of noncanonical signaling (225). Ectopic expression of both canonical (Wnt1 and -3a) and noncanonical Wnts causes changes in somite compartmentalization, and ultimately an enlarged myotome, in the chick embryo (226). Chen *et al.* utilized a comprehensive series of experiments

to demonstrate that Wnt induction of muscle-specific genes in the somite may depend on non-canonical signaling via protein kinase A (PKA) and the transcription factor CREB (227). Until this study, these signal transducers had not been implicated as Wnt pathway components.

A recent study highlights the potential role of Wnt signaling in muscle regeneration and conversion to the myogenic lineage. It was reported that expression of Wnts 5a, 5b, 7a, and 7b is upregulated in regenerating tibialis anterior muscle following cardiotoxin injury. This seems to occur through β-catenin, as its expression is increased during regeneration. Both stimulation of canonical Wnt signaling by LiCl and co-culture with Wnt-expressing cells have the ability to convert muscle-derived hematopoietic stem cells ($CD45^+$:$Sca1^+$) into myogenic cells. Injection of sFRPs into regenerating muscle significantly decreases the conversion of stem cells to myogenic cells (228). Recently, the potential for $CD45^+$ stem cells to differentiate into cells of the myogenic lineage has been called into question. Instead, it is suggested that the rare bone marrow-derived stem cells in muscle do not undergo myogenic conversion, and that skeletal muscle regeneration is due almost entirely to satellite cells (229). In addition, Zhao and Hoffman (230) report no increase in any of the Wnts mentioned above during regeneration of gastrocnemius muscles, and cite significant increases in expression of several of the sFRPs. They suggest that the *in vitro* experiments performed by Polesskaya *et al.* may be more representative of development than regeneration.

In reserve cells, the mononucleated cells that remain undifferentiated in myotube culture, canonical Wnt signaling acts in combination with insulin to activate

cells by induction of MRF expression. In cells that have already differentiated into myotubes, the result is increased fusion of reserve cells, leading to myotube hypertrophy (231). Since reserve cells, like satellite cells, express both Myf5 and CD34, this suggests the possibility of a role for Wnt signaling in satellite cell activation and skeletal muscle differentiation (65, 231, 232).

The potential roles of Wnt inhibitors during myogenesis have also been examined. Frzb1/sFRP3 strongly inhibits myogenesis in explanted early mouse somites, although older somites exhibit the normal pattern of muscle-specific gene expression. *In vivo* overexpression by placental injection of Frzb1-expressing cells has the ability to severely impair mesodermal development and the progression of myogenesis (233). In addition, sFRP2 overexpression in the chick wing bud has an inhibitory effect on myoblast differentiation (234). It is hypothesized that sFRP2 may also be involved in the activation of satellite cells and muscle regeneration. Although it is expressed in rabbit muscles during development, it is no longer present by five days after birth. However, its expression is rapidly induced following muscle injury, an effect which may be attributable to satellite cell activation. It is also expressed at relatively high levels in isolated satellite cells in culture (235). Together, these results indicate that a number of components of the Wnt pathway are vital for the normal determination, development, and maintenance of skeletal muscle.

The aims of this study were: 1) To determine differences in gene expression between myostatin-null and wild-type mice, and 2) To utilize this information to identify factors that may be downstream targets of myostatin signaling. This

research is necessary for increasing our understanding of the mechanism through which myostatin acts to inhibit skeletal muscle growth.

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CHAPTER 2. TRANSCRIPTIONAL PROFILING OF MYOSTATIN-KNOCKOUT MICE IMPLICATES WNT SIGNALING IN POSTNATAL SKELETAL MUSCLE GROWTH AND HYPERTROPHY

A paper to be submitted to the FASEB Journal for online publication

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Abstract

Myostatin is an inhibitor of skeletal muscle growth. Disruption of the *Mstn* gene in mice results in muscles that weigh 2-3 times those of controls, but precisely how myostatin signals to exert its effects on muscle is unclear. We utilized the Affymetrix GeneChip system to identify differences in gene expression between myostatin-null and wild-type mice. The results indicated a switch in muscle fiber type, from slow to fast, in the absence of myostatin. They also suggested that myostatin may act upstream of Wnt pathway components. Additional experiments supported the hypothesis that myostatin acts as an inhibitor of Wnt4, leading to a decrease in skeletal muscle satellite cell proliferation. This evidence points to a role for Wnt/calcium signaling in the growth and maintenance of postnatal skeletal muscle. This study offers new insight into potential downstream targets of myostatin, which will be beneficial for elucidation of the mechanism through which myostatin acts to inhibit muscle growth.

Introduction

The ability to increase skeletal muscle mass is highly desirable for both human health and agricultural applications. Of major interest in this pursuit is the

growth factor myostatin (GDF-8). A member of the transforming growth factor (TGF)-β superfamily, myostatin acts as a specific inhibitor of skeletal muscle growth. Mice with a targeted mutation in the *Mstn* gene exhibit a significant increase in muscle mass, with some muscles up to 2-3 times larger than those of controls (1). Early in the 19th century, a similar phenotype was first described in cattle (2). Double-muscled animals of breeds such as the Belgian Blue and Piedmontese exhibit an approximate 20% increase in skeletal muscle mass (3), which has been attributed to mutations of the myostatin gene (4-6). Recently, the first report of human muscle hypertrophy resulting from a myostatin mutation was issued (7).

Myostatin's expression is almost entirely confined to skeletal muscle. During mouse development, it is present in the somites by 9.5 days post-coitus (d.p.c.), and it continues to be expressed throughout gestation and into adulthood (1).

Myostatin is believed to inhibit muscle growth by negatively regulating both myoblast proliferation (8-12) and differentiation (8, 13, 14). It has been shown to bind to activin receptor type IIB (ActRIIB) at the cell surface (15, 16), which then recruits and activates a type I receptor, activin receptor-like kinase 4 (ALK4) or ALK5. This leads to phosphorylation and activation of SMADs 2 and 3 (13, 16, 17), which translocate into the nucleus and function as transcriptional co-modulators (reviewed in 18). An alternative pathway independent of the SMADs has also been proposed (19). Despite these advances, the precise mechanism whereby myostatin acts to repress muscle growth is unknown.

The examination of models of muscle hypertrophy can provide valuable insight into ways in which it may be possible to genetically or pharmacologically

increase skeletal muscle mass. Myofiber hypertrophy is largely dependent upon satellite cells. Satellite cells are quiescent, mononucleated cells located at the periphery of the muscle fiber (20). Upon activation, they begin to proliferate and contribute to postnatal muscle growth or repair by fusing with each other or with existing fibers (21-23). In addition, a number of signaling molecules are implicated in hypertrophy. The potential for cross-talk between signaling pathways increases the complexity of the hypertrophic response.

The goal of this study was to identify differences in gene expression between wild-type and myostatin-null mice at 3 developmental time points using the Affymetrix Mouse Expression Set 430. Two embryonic time points, 13.5 and 17.5 d.p.c., were chosen because they follow the two major waves of myogenesis that occur during development (24). In addition, muscle from mice at 5 weeks of age was examined. Based on the results of microarray analysis, we hoped to gain insight into factors that may be capable of interacting with or being influenced by myostatin. This information is critical in order to further elucidate the specific mechanism through which myostatin signals to inhibit the growth of skeletal muscle.

Materials and Methods

Animals

Animals were housed in a controlled environment on a 12/12 hour light/dark cycle and given *ad libitum* access to food and water. All animals were treated in accordance with the guidelines of the Iowa State University Animal Care and Use Committee. Myostatin-null mice (C57BL/6J background) were obtained from MetaMorphix, Inc. (Baltimore, MD). These mice were used for microarray analysis.

Mice with a transgene for nuclear localized β-Galactosidase inserted into the *Myf5* locus ($Myf5^{nlacZ/+}$) were obtained from Michael Rudnicki (Ottawa Health Research Institute, Ottawa, Ontario). This transgene allows for detection of myogenic cells by X-Gal staining (25, 26). In the adult mouse, expression is limited to satellite cells (27, 28). The $Myf5^{nlacZ}$ allele was backcrossed into the myostatin line to allow identification of satellite cells by staining for β-galactosidase. Myostatin/$Myf5^{nlacZ}$ mice are propagated by heterozygous matings.

Embryo collection

Timed-pregnant female mice at either 13.5 or 17.5 days of gestation were euthanized by CO_2 asphyxiation, and embryos were collected immediately. Whole embryos (13.5 d.p.c.) or hindquarters only (17.5 d.p.c.) were flash-frozen in liquid nitrogen and stored at -80°C until RNA isolation.

Muscle collection

Myostatin-null and wild-type C57BL/6J mice were genotyped at three weeks of age. At five weeks of age, mice were killed by cervical dislocation. The pectoralis muscle was isolated, flash-frozen in liquid nitrogen, and stored at -80°C until RNA isolation.

RNA isolation

Frozen tissue was immersed in RLT lysis buffer (Qiagen, Valencia, CA) and homogenized for 45 seconds using a Brinkman Polytron homogenizor. Lysates were centrifuged at 3600xg for 10 minutes to pellet cell debris. Total RNA was isolated from the lysates using the RNeasy Midi Kit (Qiagen) with on-column DNase digestion, according to the manufacturer's protocol. For isolation of RNA from

pectoralis muscle, the RNeasy protocol for heart, muscle, and skin tissue was used, which includes digestion with Proteinase K (Sigma, St. Louis, MO) for 20 minutes at 55°C. Isolated RNA was quantified based on absorbance at 260 nm, and quality was assessed by agarose gel electrophoresis. For use in microarray analysis, 10 µg of RNA from each of three individual animals was pooled to give five pools for each genotype/time point combination. The pooled RNA was diluted in RNase-free water to yield a final concentration of 1 µg/ml.

Target preparation

Microarray target preparation, hybridization, and scanning were carried out at the Iowa State University GeneChip Facility. Double-stranded cDNA was synthesized from pooled RNA using the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). T7-oligo(dT) primer and SuperScript II reverse transcriptase were used in first-strand synthesis. Biotin-labeled cRNA was generated with the BioArray High Yield RNA Transcript Labeling Kit (Enzo Life Sciences, Farmingdale, NY). cDNA and cRNA cleanup, as well as fragmentation of cRNA, were carried out using the GeneChip Sample Cleanup Module (Qiagen, distr. by Affymetrix). Quality of cDNA, cRNA, and fragmented cRNA was confirmed using the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

Microarray analysis

Target RNA was hybridized to both the A and B chips of the GeneChip® Mouse Expression Set 430 (Affymetrix, Santa Clara, CA), which encompasses over 39,000 sequences (genes and ESTs) from the mouse transcriptome. Hybridization and scanning were carried out according the GeneChip Expression Analysis

Technical Manual, available at

www.affymetrix.com/support/technical/manual/expression_manual.affx.

Examination of raw data was performed using the Microarray Suite 5.0 software package (Affymetrix). All chips met the following criteria: 1) Background less than 300; 2) Noise less than 10; and 3) ratio of 3'/5' hybridization less than 3 for β-actin housekeeping gene.

Statistical analysis

Data were normalized to a default target signal value of 500 using the scaled probe method in the Microarray Suite software. Gene expression data were analyzed as a completely randomized design with 5 replicates for each genotype and day combination. The variances were unequal across time points. Because of this, the analysis was performed on PROC MIXED using SAS version 8.2 (SAS Inc., Carey, NC). The model used in analyzing the data was $y_{ijk} = \mu + G_i + D_j + GD_{ij} + \varepsilon_{ijk}$. Genotype is indicated by i, day by j, and replication by k. Because of the differences in variability, ε_{ijk} is assumed to have a different normal distribution at each day.

Annotation of genes by EASE

A downloadable software tool, Expression Analysis Systematic Explorer (http://david.niaid.nih.gov/david/ease.htm) was used to categorize genes found to be differentially expressed by microarray. Gene ontology annotation for biological process was determined by linking to the Database for Annotation, Visualization, and Integrated Discovery (DAVID, http://david.niaid.nih.gov/david/) (29). EASE was used to find categories that were overrepresented in groups of up- or downregulated genes at each time point (30).

Real-time PCR

cDNA was synthesized from 4 µg of individual animal RNA using an oligo(dT) primer and the protocol for First-Strand cDNA Synthesis Using SuperScript III for RT-PCR (Invitrogen). cDNA was diluted 1:4 in nuclease-free water. Real-time PCR reactions (2 µl diluted cDNA per reaction) were run on a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA) using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Gene-specific primers were used to amplify cDNA from 15 myostatin-null and 15 wild-type muscle samples, each in duplicate. Primer sets were as follows: Wnt4: 5'-CGCGCTAAAGGAGAAGTTTG-3'/ 5'-CGGGCTAGGCTCCAGGTAT-3', sFRP1: 5'-CGCCCGTCTGTCTGGACCG-3'/ 5'-CTCGCTTGCACAGAGATGT-3', Catnbip1: 5'-ATCTGGTCCCTCAGCACATC-3'/ 5'-AGCAATGACGCTCTCCTCTC-3', Camk2d: 5'-GCATACATTCGGCTCACACA-3'/ 5'-ATTCTGCCACTTCCCATCAC-3', β-actin: 5'-GACAGGATGCAGAAGGAGATTACT-3'/ 5'-TGATCCACATCTGCTGGAAGGT-3'. The PCR program consisted of denaturation for 10 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. Fold-change in gene expression between genotypes was determined using the ΔΔCt method (31), with samples normalized to the level of β-actin expression.

Cell Culture and Transfection

HEK 293 cells and COS7 cells (American Type Culture Collection, Manassas, VA) were maintained in a humidified incubator at 37°C, 5% CO_2. Unless indicated otherwise, reagents were purchased from Gibco (Grand Island, NY), Atlanta Biologicals (Lawrenceville, GA), or Fisher Scientific (Pittsburgh, PA). Culture media

consisted of DMEM plus 10% fetal bovine serum, 2 mM L-Glutamine, and 1.0 mg/ml penicillin/streptomycin. For transfections, culture media without antibiotics was used. On the day prior to transfection, HEK 293 cells were plated at a density of 10,000 per cm^2 on 6-cm culture dishes. Cells were transfected with 2 µg plasmid DNA using Lipofectamine 2000 (Invitrogen). COS7 cells used for immunocytochemistry were plated at 5000 cells/cm^2 on bovine fibronectin-coated glass coverslips in a 12-well plate and transfected with 0.3 µg of plasmid DNA. pLNCX-HA and HA-Wnt4 plasmids were kindly provided by Jan Kitajewski (Columbia University, New York, NY). pCMV-HA and HA-sFRP1 and -2 were gifts of Hiromo Suzuki (Sapporo Medical University, Sapporo, Japan).

Immunoprecipitation and Western Blot

Transfected HEK 293 cells were lysed in a buffer of 20 mM Tris, pH 8.0, 2 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1 mM phenymethylsulphonyl fluoride, 10 µg/ml aprotinin, and 10 µg/ml leupeptin. Following a brief incubation on ice, they were scraped from the plates. Lysates were centrifuged for 3 minutes at 3000xg to pellet cell debris. HA-tagged proteins were immunoprecipitated from the supernatant using 1 µg/ml of rabbit anti-HA antibody (Santa Cruz, Santa Cruz, CA) with incubation overnight at 4°C with constant agitation. Primary antibody and bound protein were precipitated by incubation for 2 hours with PANSORBIN cells (Calbiochem, San Diego, CA). Following centrifugation, the cell pellet was washed 3 times with lysis buffer. The pellet was resuspended in SDS-PAGE loading buffer and incubated for 20 minutes at 55°C. The PANSORBIN cells were pelleted by brief centrifugation, and the supernatant was subjected to SDS-PAGE with 5% stacking

and 12% resolving gels. Protein was transferred to a Westran PVDF membrane (Schleicher & Schuell BioScience, Keene, NH). The membrane was blocked with 5% BSA and incubated for 2 hours in 1:1000 mouse anti-HA antibody (Covance, Berkeley CA) in PBS-T. Following 2 washes in PBS-T, an incubation was carried out with 1:5000 goat anti-mouse, peroxidase-conjugated secondary antibody (Sigma). Protein was detected using the Amersham ECL Plus kit (Piscataway, NJ) and visualized on a FluorChem 8800 imager (Alpha Innotech, San Leandro, CA).

Immunocytochemistry

Transfected COS7 cells were fixed for 30 minutes in 1% formaldehyde, 0.5% sucrose in PBS. They were then rinsed and incubated in 1% IGEPAL for 4 minutes. Coverslips were incubated in blocker (5% goat serum, 0.4% BSA, and 0.2% Triton X-100 in PBS) for 20 minutes, then placed in 1:500 anti-HA antibody (Santa Cruz) in PBS overnight at 4°C. Cells were rinsed and incubated for 3 hours in 1:500 anti-rabbit secondary antibody (Molecular Probes, Eugene, OR) and 1 µg/ml Hoechst (HO33258). Coverslips were mounted on slides and imaged using a Leica DMIRE2 microscope with OpenLab software (Improvision Inc., Lexington, MA).

Satellite Cell Isolation

Satellite cells were isolated from four-month-old $Myf5^{nlacZ/+}$;$Mstn^{+/+}$ and $Myf5^{nlacZ/+}$;$Mstn^{-/-}$ mice using a protocol modified from Chakravarthy *et al.* (32). Mice were killed by CO_2 asphyxiation, and as much skeletal muscle as possible was collected. The tissue was rinsed thoroughly with sterile PBS and chopped finely using sterile razor blades. The muscle was then digested in a 1.2 mg/ml solution of Protease (Type XIV, Sigma) at 37°C for 40 minutes and vortexed every 10 minutes

throughout. Following digestion, samples were centrifuged for 15 minutes at 1200xg, and the supernatant was removed. The muscle tissue was resuspended in DMEM and the centrifugation repeated. Muscle was again resuspended in DMEM and subjected to centrifugation at 250xg. This step was repeated an additional two times, and the supernatants were pooled. The pooled supernatant was then centrifuged at 1200xg, and the cell pellet was resuspended in growth media, which consisted of Ham's F-10 plus 20% fetal bovine serum, 2% penicillin/streptomycin, 0.002% Fungizone, and 2.5 ng/ml bFGF (Invitrogen). The suspension was filtered through sterilized nylon mesh and plated on 12-well plates coated with Vitrogen collagen (Cohesion Technologies, Palo Alto, CA). Cultures were maintained in a humidified CO_2 incubator under low-oxygen conditions (~3% O_2), which have been utilized with satellite cells previously since they more closely mimic *in vivo* levels (33, 34).

Co-culture

Isolated satellite cells were plated at a density equivalent to approximately 185 milligrams of tissue per cm^2. The cells were allowed to attach for 48 hours, at which time the culture media was replaced, and HEK 293 cells that had been transfected the previous day with HA-Wnt4, HA-sFRP1 and-2, or the corresponding empty vector were added (1850 cells per cm^2). The cells were co-cultured for 48 hours, then fixed and stained. During the final 4 hours of co-culture, cells were incubated with 10 μM BrdU and dCTP.

X-Gal Staining

Following a PBS rinse, cells were fixed in 2% formaldehyde for 10 minutes. Cultures were then rinsed 3 times in PBS and stained for 2 hours in a solution of 0.5 mg/ml X-Gal, 4 mM potassium ferricyanide, 4 mM potassium ferrocyanide, 2 mM $MgCl_2$, and 0.1% Tween-20 in PBS. During staining, the cells were incubated at 37°C in a humidified CO_2 incubator. Stained cells were rinsed thoroughly and post-fixed for 10 minutes in 2% formaldehyde.

Detection of BrdU

Following X-Gal staining, cell proliferation was assayed by antibody detection of incorporated BrdU. Post-fixed cells were rinsed twice with PBS, then incubated for 15 minutes with 4M HCl. The cells were rinsed again with PBS and placed in a solution of 1% IGEPAL for 4 minutes. Following 2 washes in PBS containing 0.1% Tween-20 (PBS-T), cells were incubated overnight at 4°C in BrdU primary antibody (Developmental Studies Hybridoma Bank, Iowa City, IA) diluted 1:125 in PBS-T plus 1% bovine serum albumin. Cells were washed with PBS-T and incubated for 1 hour in 1:500 anti-mouse, FITC-conjugated secondary antibody (Sigma). Cells that had stained positive for β-galactosidase were assessed for BrdU incorporation using a Nikon fluorescent microscope. Double staining of satellite cells is pictured in **Fig. 1**. The percentage of proliferating cells was determined by dividing the number of cells that were BrdU positive by the total number of β-Galactosidase-positive cells.

Results

Muscle weights

Pectoralis muscles obtained from 5-week-old myostatin-null and wild-type mice were weighed prior to RNA isolation. The average mass of myostatin-null

muscle (108.9±6.9 mg) was found to be significantly greater than that of wild-type (72.6±5.9 mg; $P<0.001$).

Microarray analysis

Microarray analysis revealed a substantial number of differences in gene expression between myostatin-null and wild-type mice. Because the samples collected at each of the time points were different (whole embryo at 13.5 d.p.c., hindquarters at 17.5 d.p.c., and pectoralis muscle at 35 days of age), it was not possible to make comparisons across time. Therefore, the analysis focused on differences within a given time point. Initially, a Bonferroni correction was done to obtain a conservative value for the number of genes that were differentially expressed. The results were as follows: 13.5 d.p.c.: 30 genes, 17.5 d.p.c.: 11 genes, and day 35: 200 genes. Data were then re-analyzed to allow for a false discovery rate (FDR) of 0.05. From FDR analysis, the smallest number of differentially expressed genes was identified at 17.5 d.p.c. (73 genes) and the largest number at 35 days of age (2109 genes), with 472 genes at 13.5 d.p.c.

EASE analysis

Genes found to be differentially expressed between myostatin-null and wild-type mice were further analyzed using EASE. Examination of the GO: Biological Process annotation of the genes revealed that each of the time points had categories in common. Metabolism, cell communication, morphogenesis, and response to stimulus were among the classifications which composed a notable percentage of the genes at all three time points (**Fig. 2**). Statistically

overrepresented biological process categories were also determined for groups of up- or downregulated genes (**Table 1**).

Changes in Wnt signaling

Initial assessment of the genes found to be differentially expressed (FDR) at 35 days of age indicated that there were several genes that are components of the Wnt signaling pathway. Members of both the canonical pathway, through β-catenin, and the Wnt/calcium pathway were affected (**Fig. 3**).

Real-time quantitative PCR

In order to validate changes in gene expression discovered on the microarray, real-time quantitative PCR was performed. Four genes involved in Wnt signaling were confirmed to be differentially expressed between wild-type and myostatin-null skeletal muscle (**Table 2**). The direction of change agreed with that which was indicated by the microarray. For the most part, the larger the fold-change in expression observed on the array, the more likely a gene was to have a significant result from real-time PCR. As has been noted in the past for GeneChip arrays (35), the magnitude of change calculated for a given gene based on real-time PCR tended to be greater than that resulting from microarray.

Satellite cell proliferation assay

Proliferation assays were performed to determine the effects of manipulation of Wnt signaling on isolated satellite cells. Since microarray and real-time PCR analysis revealed that Wnt4 is upregulated in $Mstn^{-/-}$ muscle, Wnt signaling in satellite cells from a myostatin-null mouse was inhibited through treatment with sFRP1 and -2. Conversely, Wnt signaling was stimulated in wild-type satellite cells

by incubation with Wnt4. To accomplish this, satellite cells were co-cultured with HEK 293 cells expressing the appropriate plasmid. A western blot was done to ensure that transiently transfected 293 cells were producing HA-Wnt4 (**Fig. 4A**). We were unable to confirm sFRP expression by western blot. However, expression of both sFRP1 and -2 was detected in transfected COS7 cells by immunocytochemistry (**Fig. 4B**). $Mstn^{-/-}$ satellite cells that were cultured for 48 hours with sFRP-transfected HEK 293 cells exhibited a statistically significant decrease in percent proliferation when compared to empty-vector controls ($P<0.01$). In contrast, $Mstn^{+/+}$ satellite cell cultures had an increased percentage of proliferated cells when exposed to Wnt4-expressing HEK 293 cells versus empty-vector controls ($P<0.01$) (**Fig. 5**).

Discussion

Microarray analysis of tissues from myostatin-null and wild-type mice indicated a number of differences in gene expression at all three time points examined. Ideally, comparisons would have been made using the same tissue sample from each of the time points. This would allow us to draw conclusions about changes in gene expression over time. However, obtaining single muscles from 13.5 and 17.5 d.p.c. embryos was not feasible due to their small size. Initially, a conservative estimate of the number of genes differentially expressed was obtained by Bonferroni correction. Subsequently, an additional adjustment was performed to allow a false discovery rate of 0.05, which accepts that 5% of the genes declared differentially expressed will be false positives. This approach was devised to

manage the rate of error incurred during multiple testing (36) and has been discussed previously (37).

Gene ontology annotation was used to elicit information on the classes of genes most affected by a lack of functional myostatin. Expression of genes involved in a wide variety of biological processes was altered. Changes in a number of genes involved in metabolism, morphogenesis, enzyme activity, nucleic acid or protein binding, and cell communication were present at all three time points. At either of the embryonic time points, the inclusion of multiple tissues added complexity; however, alterations in stimulus response, apoptosis, cell differentiation, and translation were apparent. The results painted a clearer picture at day 35. In pectoralis muscle, loss of myostatin impacted transcription, translation and protein modification, growth, differentiation, tissue organization, homeostasis, and energy production. All of these changes would be expected with enhanced muscle growth.

For two reasons, further analysis of microarray data focused on the results from day 35. First of all, this day had the largest number of affected genes. More importantly, it was the only time point at which exclusively skeletal muscle was used, and myostatin expression and activity are largely confined to this tissue.

One notable finding in the day 35 data was the substantial number of muscle-specific genes that were differentially expressed. Specifically, the results indicated a switch in muscle fiber type. Myostatin-null tissue had decreased expression of myosin and troponin genes contributing to the slow phenotype. A similar observation has been made by Girgenrath, *et al.* (38), who reported that myostatin-null mice have an increase in the number of fast, glycolytic muscle fibers at the

expense of slow, oxidative fibers. Past research has also suggested that myostatin is preferentially expressed in fast-twitch fibers in mice (39). This data is supported by studies on double-muscled cattle (40, 41). Since our microarray data did not indicate increased expression of genes for fast protein isoforms, we believe any upregulation of the fast phenotype may be attributable to post-transcriptional events. This is in agreement with the results of EASE analysis, in which translation was determined to be an overrepresented functional category among the upregulated genes.

Upon examination of the list of genes differentially expressed at day 35, it was noted that a number of genes involved in Wnt signaling were represented. This was later verified by EASE analysis of functional annotation, in which the gene category was determined to be statistically overrepresented (EASE score ~0.08-0.17). It appears that loss of myostatin may result in downregulation of Wnt signaling through β-catenin, and upregulation of the Wnt/calcium pathway. TGF-β has previously been shown to act upstream of Wnt pathway components (42, 43). Wnt4 is believed to act only through the Wnt/calcium pathway, as it fails to meet the major criteria for inclusion in the class of Wnts that activates β-catenin (44, 45). It has been demonstrated that the members of the group of Wnts that activates calcium signaling can act as antagonists to those that signal through β-catenin (46).

Real-time PCR was used to confirm differential expression of four genes that are components of the Wnt pathway, including Wnt4. Nine additional genes were analyzed, but the results were not statistically significant (data not shown). This is likely due to the fact that each of these genes had less than a 2-fold difference in

expression level between wild-type and myostatin-null, and real-time PCR is unable to detect differences this small.

Wnt signaling is known to play a role in embryonic myogenesis. Wnts act in concert with Sonic Hedgehog (Shh) to induce myotome formation (47). Wnt signals have been shown to be necessary and sufficient for the induction and maintenance of dermomyotome (48, 49). In fact, ectopic Wnt expression in the chick embryo results in expansion of the myotome (50, 51). In the chick wing bud, manipulation of Wnt signaling alters myoblast differentiation and fiber-type specification (52). Several Wnts, including Wnt4, are capable of inducing the myogenic regulatory genes *Myf5* and *MyoD* (53). A recent study indicates that this regulation may be a result of Wnt signaling through different pathway components, including protein kinase A and the transcription factor CREB (54). Most studies on the role of Wnt pathway components in skeletal muscle have focused on members of the canonical pathway (55-57).

A potential role for Wnts in the regeneration of skeletal muscle has been proposed. Polesskaya *et al.* reported that stimulation of canonical Wnt signaling induced a population of muscle-derived hematopoietic stem cells to adopt a myogenic fate (58). However, it is unclear whether these cells are capable of contributing to muscle regeneration, as they comprise only a very small fraction of the cell population, and their myogenicity is limited (59).

Wnts are able to induce cell proliferation not only during myogenesis, but also in the development of the pituitary gland and cardiac outflow tract (60), as well as in

the intestinal epithelium (61-63). In addition, canonical Wnt signaling has been shown to stimulate proliferation of human mesenchymal stem cells (64, 65).

In order to identify a potential role for Wnt4 in postnatal muscle growth and hypertrophy, we examined the effects of stimulation or inhibition of Wnt4 signaling on satellite cell proliferation. Previous research has shown that myostatin is capable of suppressing satellite cell proliferation (66). Since Wnt4 was found to be upregulated in $Mstn^{-/-}$ skeletal muscle, we hypothesized that myostatin may act as an inhibitor of Wnt4. Given the ability of Wnts to stimulate cell proliferation in a number of tissues, we believed that repression of Wnt4 by myostatin may lead to a decreased rate of satellite cell proliferation in skeletal muscle. Therefore, we exposed satellite cells isolated from $Mstn^{+/+}$ mice to Wnt4, to determine if the exogenous Wnt could overcome repression by myostatin and stimulate cell proliferation. Alternatively, we exposed $Mstn^{-/-}$ satellite cells to a combination of the Wnt inhibitors sFRP1 and -2. The sFRPs are secreted proteins that exhibit homology to the Wnt-binding region of Frizzled receptors (67, 68). sFRP1 and -2 are each capable of interacting with Wnt4 (69, 70), and both have been shown to inhibit its activity (70, 71). sFRP2 has been implicated in the regulation of myogenesis (52, 72). sFRP1 and -2 were used together because both were decreased in $Mstn^{-/-}$ muscle.

In order to circumvent the problems associated with secretion of Wnt protein, satellite cells were co-cultured with HEK 293 cells that had been transfected with an HA-Wnt4 construct. Several studies have shown that the secretion of glycosylated Wnts is inefficient, with the majority of the protein either retained in the endoplasmic

reticulum or closely associated with the cell membrane (73-78). Western blot of 293 cell lysates using an anti-HA antibody indicated that transfected cells were producing Wnt4 protein. We were unable to immunoprecipitate sFRP1 or -2 protein from cell lysates, even though a previous study concluded that sFRP1 is usually associated with the cell surface (67). This may be because the sFRPs are produced by transiently transfected HEK 293 cells at levels too low to be detected by Western blot. However, both HA-sFRP1 and -2 were detected in transfected COS7 cells by immunocyotchemistry, indicating that the constructs are functional.

Effects of Wnt4 or sFRP1 and -2 exposure on satellite cell proliferation were assessed by comparison to empty vector controls. Only data on β-Galactosidase-positive cells was collected, to be certain that we were limiting our analysis exclusively to satellite cells. Use of this method may result in a small subset of the satellite cells being disregarded (27, 28), but it ensures that fibroblasts or co-cultured 293 cells are not inadvertently included.

Our results showed that co-culture with Wnt4-expressing cells increased the proportion of proliferated $Mstn^{+/+}$ satellite cells by approximately 15%. Conversely, $Mstn^{-/-}$ satellite cell cultures containing sFRP-transfected cells had 15% fewer cells proliferate than controls. Conclusions about the proliferation of wild-type versus myostatin-null satellite cells cannot be drawn from this assay. This data indicates that Wnt4 stimulates the proliferation of satellite cells, while inhibition of Wnt signaling downregulates cell proliferation.

This study identified a large number of genes that were differentially expressed between myostatin-null and wild-type mice. It also provided evidence of

a role for Wnt signaling in the growth and maintenance of postnatal skeletal muscle, specifically in satellite cell proliferation. We propose a model in which Wnt4 is inhibited in the presence of myostatin, resulting in a decrease in satellite cell proliferation. Alternatively, upregulation of Wnt4 in the absence of myostatin leads to stimulation of satellite cell proliferation. Our results offer new insight into genes which may interact with myostatin to regulate skeletal muscle growth. Further analysis of these genes will be valuable in the quest to elucidate the precise mechanism by which myostatin signaling occurs.

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Figure and Table Legends

Figure 1. Double staining of Myf5$^{nlacZ/+}$ satellite cells. *A*) β-Galactosidase^{+} cells stained with X-Gal; *B*) proliferating cells identified by staining with an antibody to BrdU. Arrows indicate cells which have not undergone proliferation. Magnification: 400x.

Figure 2. Biological process annotation of differentially expressed genes. Using DAVID, gene ontology classification was determined for up- or down-regulated genes found to be significant by FDR analysis at 13.5 d.p.c, 17.5 d.p.c,

and day 35. At day 35, "other classes" include homeostasis, reproduction, embryonic development, pattern specification, growth, epigenetic regulation of gene expression, and secretion, among others.

Figure 3. Wnt signaling pathways. The canonical Wnt pathway, through β-catenin, and the Wnt/calcium pathway are depicted. Genes in green were upregulated in myostatin-null versus wild-type mice, while those in red were downregulated. Other genes pictured were not differentially expressed. In pectoralis muscle from 35-day-old mice, expression of Wnt4, Dishevelled (Dvl), Calcineurin, CamkIIA and -D, Catnbip1, Dickkopf1 (Dkk1), sFRP5, MEF2A, and HDAC10 was found to be increased. In contrast, sFRP1 and -2, Dickkopf2 (Dkk2), and MCIP expression was decreased.

Figure 4. Expression of HA-Wnt4 and HA-sFRP1 and -2. *A*) Representative western blot showing expression of HA-Wnt4 in transfected 293 cells. HA-tagged proteins were immunoprecipitated from cell lysates and electrophoresed on a 12% polyacrylamide denaturing gel. Lane 1, empty vector (pLNCX-HA); lane 2, HA-Wnt4. *B*) Visualization of sFRPs in transfected COS7 cells by immunocytochemistry. HA-sFRP1 and -2 proteins were detected using an antibody to hemaglutinnin, and nuclei were visualized using Hoechst. Magnification: 400x.

Figure 5. Effects of Wnt4 and sFRP exposure on satellite cell proliferation. Percent proliferation is the mean percentage (± SEM) of cells positive for BrdU incorporation in *A*) myostatin-null satellite cells incubated with 293 cells transfected with empty vector (pLNCX-HA) or HA-Wnt4; or *B*) wild-type satellite cells incubated

with 293 cells transfected with empty vector (pCMV-HA) or HA-sFRP1 and -2. *P<0.01. Results are representative of two replicates of each experiment.

Table 1. Overrepresented gene categories identified by EASE analysis of microarray data. Genes determined to be significantly different between myostatin-null and wild-type mice (5% FDR) were uploaded into EASE. Overrepresented gene categories are determined by taking into account the proportion of genes in the differentially expressed subset that are annotated for a certain biological process, given the proportion found in the entire population (i.e., all genes on the microarray). 8151 genes on the MOE430 GeneChips were annotated for GO biological process. Pop. Hits denotes the number of these genes in each functional category. Parentheses indicate number of genes in the analyzed subset that belong to the gene category versus the number annotated for any biological process. The most highly overrepresented gene categories are listed, although some redundant categories have been omitted. Others that may be of particular interest are included. The EASE score is the p-value resulting from a conservative modification of the Fisher exact test.

Table 2. Differentially expressed genes confirmed by real-time PCR. Four genes identified as differentially expressed by microarray analysis at day 35 were confirmed by quantitative real-time PCR. Fold-change is equal to the ratio of gene expression in myostatin-null to that of wild-type, as determined using each of the two methods. *Genes represented by more than one probe set on the array; data presented correspond to the result with the most significant p-value.

Figure 1

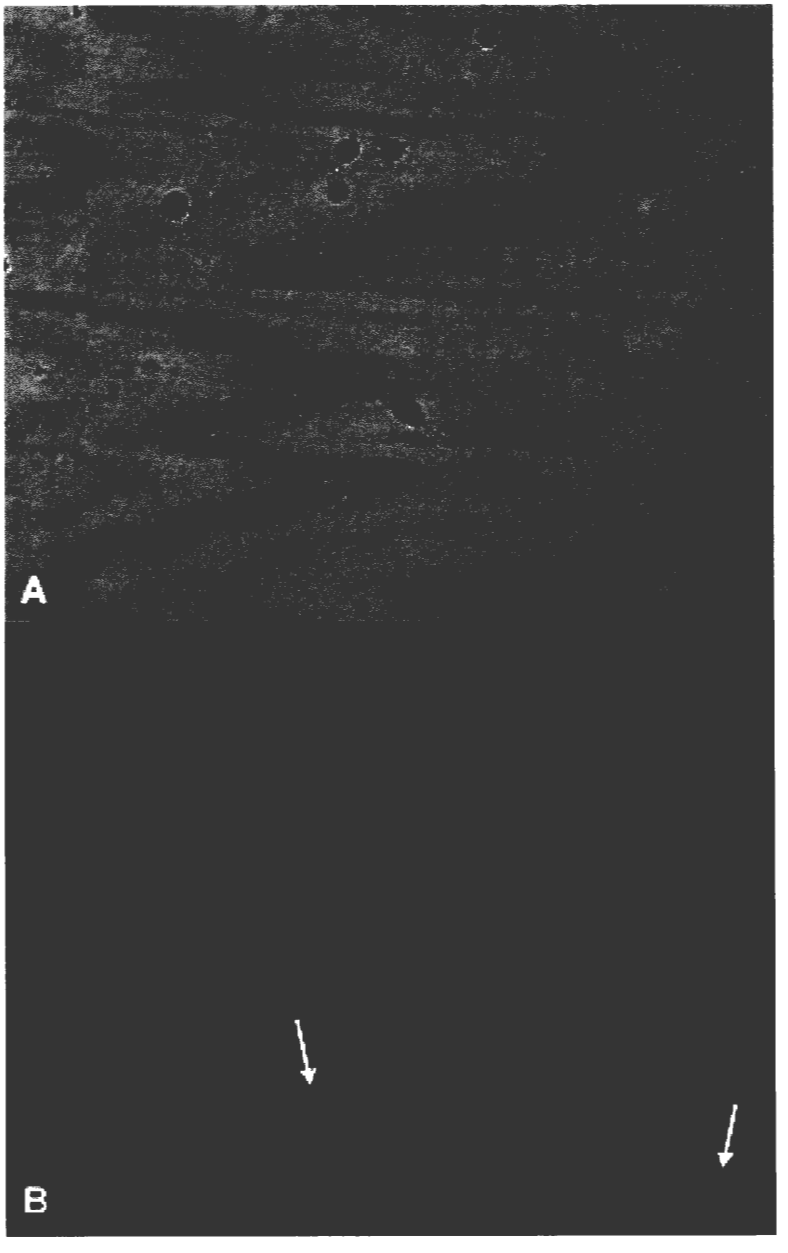


Figure 2

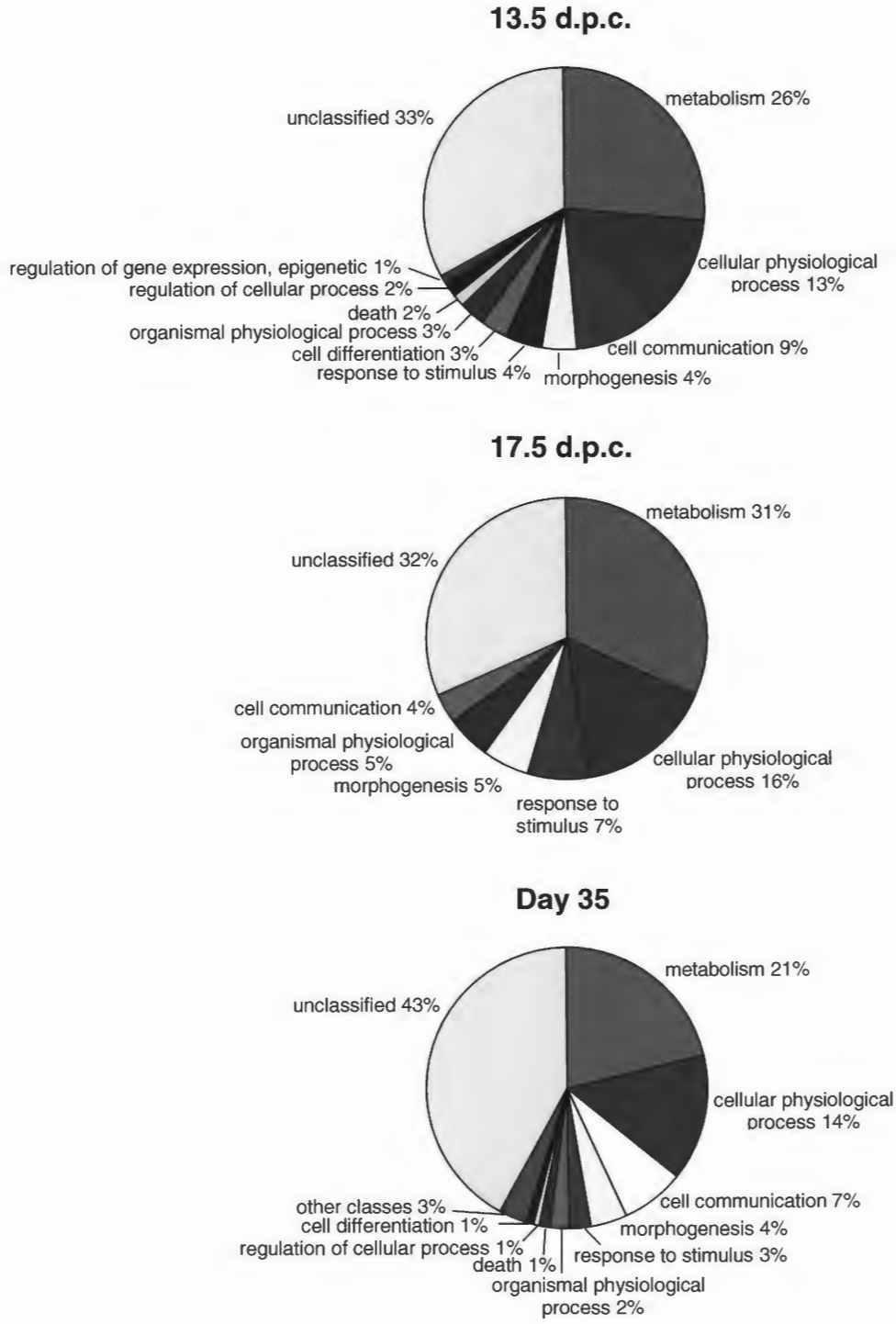
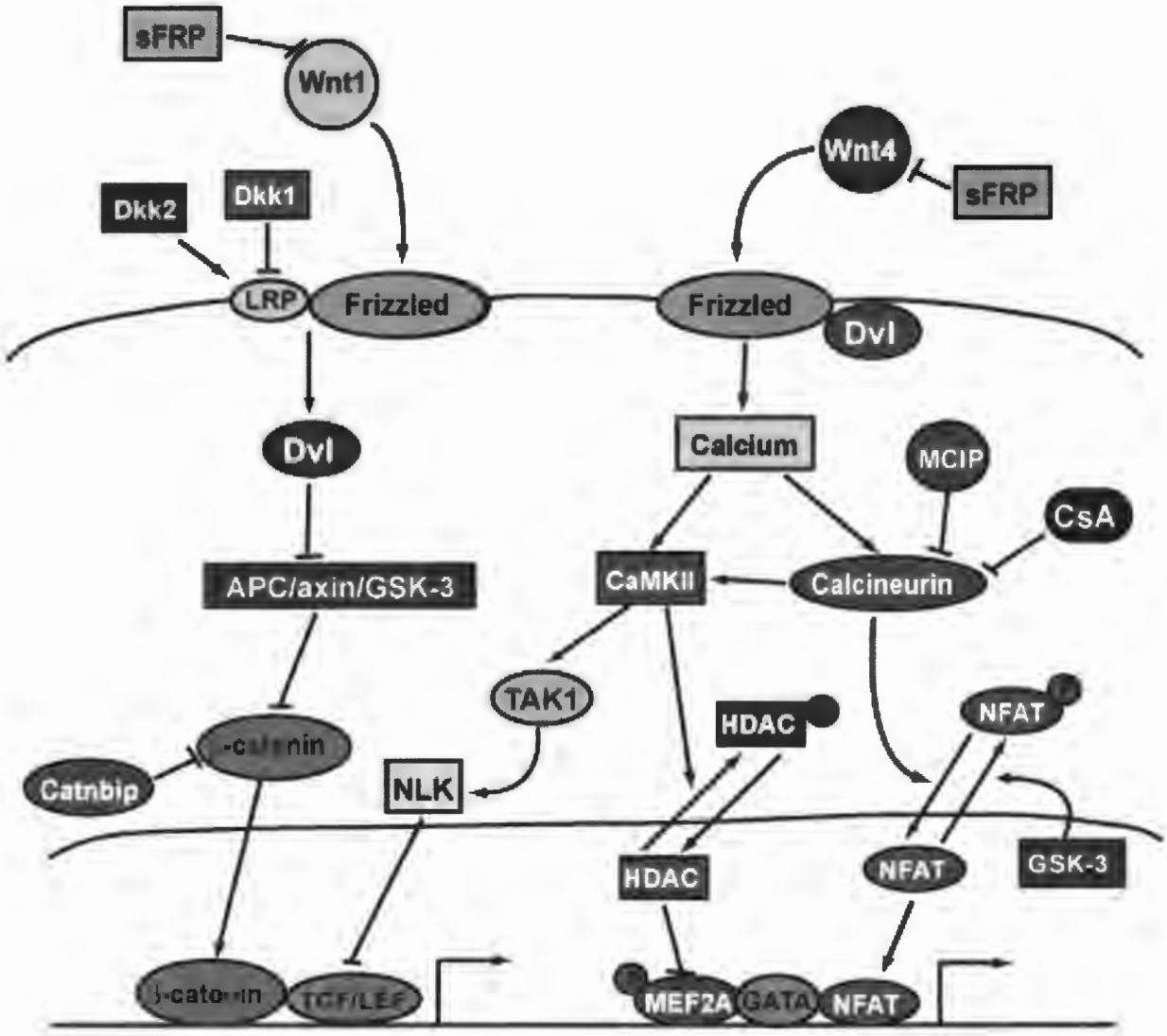

13.5 d.p.c.
metabolism 26%
unclassified 33%
regulation of gene expression, epigenetic 1%
regulation of cellular process 2%
death 2%
organismal physiological process 3%
cell differentiation 3%
response to stimulus 4%
morphogenesis 4%
cell communication 9%
cellular physiological process 13%
17.5 d.p.c.
metabolism 31%
unclassified 32%
cell communication 4%
organismal physiological process 5%
morphogenesis 5%
response to stimulus 7%
cellular physiological process 16%
Day 35
metabolism 21%
unclassified 43%
other classes 3%
cell differentiation 1%
regulation of cellular process 1%
death 1%
organismal physiological process 2%
response to stimulus 3%
morphogenesis 4%
cell communication 7%
cellular physiological process 14%

Figure 3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Wnt1
Dkk2
Dkk1
LRP
Frizzled
Wnt4
sFRP
Frizzled
Dvl
Dvl
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APC/axin/GSK-3
CaMKII
Calcineurin
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TCF/LEF
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GATA
NFAT

Figure 4

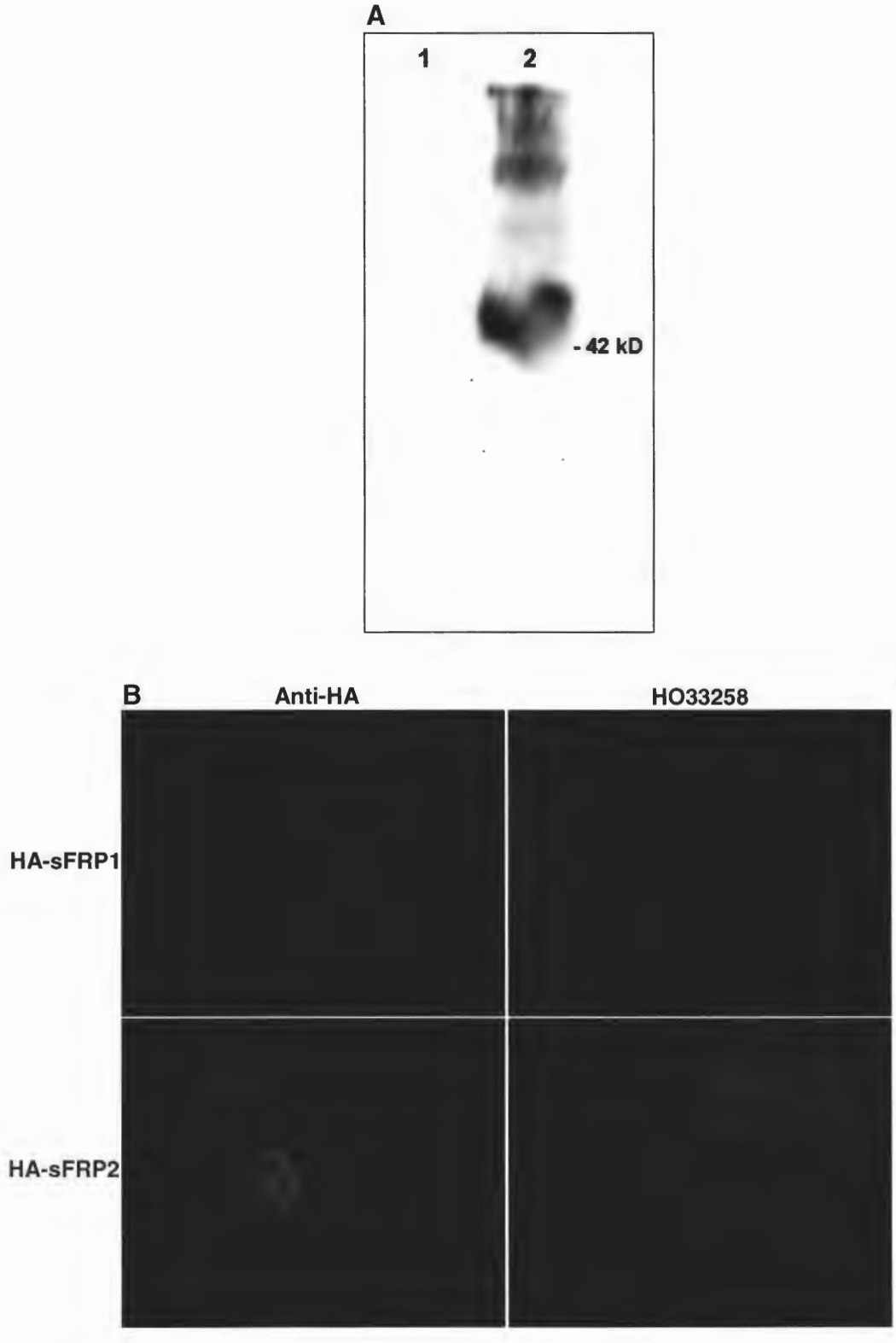

Figure 5

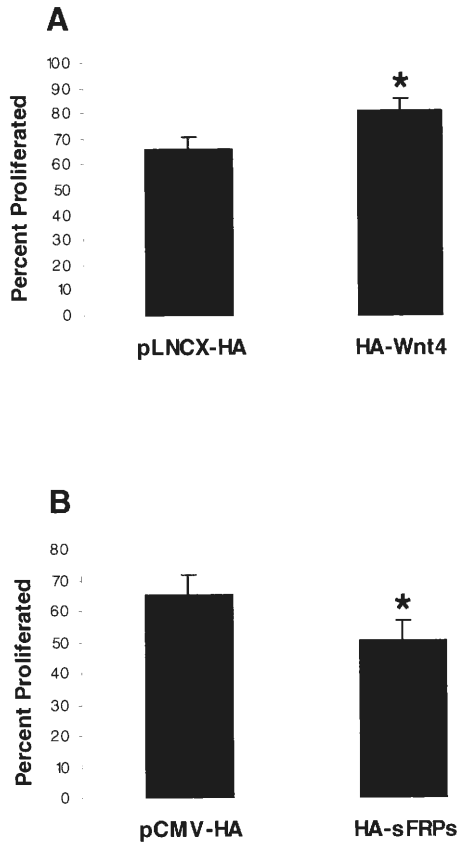
A
Percent Proliferated
100
90
80
70
60
50
40
30
20
10
0
*
pLNCX-HA
HA-Wnt4
B
Percent Proliferated
80
70
60
50
40
30
20
10
0
*
pCMV-HA
HA-sFRPs

Table 1

Upregulated genes			Downregulated genes		
Gene Category	Pop. Hits	EASE score	Gene Category	Pop. Hits	EASE score
Day 13.5					
amine metabolism (5/69)	189	7.26E-02	regulation of biological process (26/65)	1956	5.10E-03
phosphoinositide metabolism (2/69)	11	8.81E-02	regulation of cellular process (24/65)	1792	7.39E-03
cellular process (64/69)	7067	9.56E-02	phosphorylation (9/65)	416	1.55E-02
induction of apoptosis (3/69)	69	1.13E-01	regulation of physiological process (22/65)	1810	3.28E-02
cell differentiation (7/69)	416	1.32E-01	negative regulation of metabolism (5/65)	134	2.08E-02
glycerophospholipid metabolism (2/69)	18	1.40E-01	protein amino acid phosphorylation (8/65)	374	2.65E-02
positive regulation of apoptosis (3/69)	81	1.46E-01	development (18/65)	1346	2.76E-02
DNA alkylation (2/69)	20	1.54E-01	nucleotide and nucleic acid metabolism (23/65)	1893	2.79E-02
amino acid and derivative metabolism (4/69)	164	1.57E-01	transcription (17/65)	1252	2.97E-02
biopolymer metabolism (13/69)	1060	1.67E-01	primary metabolism (40/65)	3975	3.34E-02
Day 17.5					
macromolecule biosynthesis (3/10)	393	6.67E-02	protein deubiquitination (2/17)	7	1.37E-02
primary metabolism (8/10)	3975	7.82E-02	immune response (4/17)	380	3.58E-02
cellular metabolism (8/10)	4147	9.87E-02	defense response (4/17)	471	6.12E-02
nucleotide and nucleic acid metabolism (5/10)	1893	1.34E-01	response to biotic stimulus (4/17)	543	8.61E-02
metabolism (8/10)	4408	1.37E-01	organismal physiological process (5/17)	895	9.00E-02
cellular biosynthesis (3/10)	627	1.48E-01	response to stimulus (5/17)	992	1.21E-01
cell organization and biogenesis (3/10)	655	1.59E-01	protein catabolism (3/17)	421	1.99E-01
cellular physiological process (9/10)	6020	2.74E-01	biopolymer catabolism (3/17)	424	2.01E-01
macromolecule metabolism (4/10)	1965	3.74E-01	physiological process (16/17)	6767	2.17E-01
transcription (3/10)	1252	4.13E-01	macromolecule catabolism (3/17)	478	2.41E-01
Day 35					
protein metabolism (111/360)	1750	2.06E-05	fatty acid metabolism (19/324)	100	5.76E-08
macromolecule metabolism (120/360)	1965	4.69E-05	lipid metabolism (34/324)	337	1.20E-06
protein amino acid phosphorylation (33/360)	374	2.21E-04	organic acid metabolism (26/324)	271	6.78E-05
translation (14/360)	95	2.48E-04	carboxylic acid metabolism (26/324)	271	6.78E-05
phosphorylation (34/360)	416	6.94E-04	regulation of muscle contraction (5/324)	18	4.76E-03
cellular physiological process (291/360)	6020	1.05E-03	cofactor metabolism (13/324)	135	7.16E-03
amino acid activation (7/360)	31	2.02E-03	sulfur metabolism (7/324)	44	7.32E-03
tRNA aminoacylation (7/360)	31	2.02E-03	electron transport (19/324)	243	7.80E-03
phosphorus metabolism (37/360)	503	2.51E-03	muscle contraction (7/324)	49	1.23E-02
protein modification (58/360)	898	2.61E-03	muscle development (10/324)	96	1.35E-02
muscle development (10/360)	96	2.51E-02	generation of precursor metabolites & energy (24/324)	359	1.54E-02

Table 2

	Quantitative PCR		Affymetrix Microarray	
Gene	**Fold-change**	**p-value**	**Fold-change**	**p-value**
Wnt4	2.16	2.56E-02	1.90	1.82E-06*
Catnbip1	4.76	4.40E-03	2.12	8.27E-07*
sFRP1	0.63	8.69E-02	0.29	1.31E-04
Camk2d	3.30	7.00E-04	1.38	3.52E-03*

CHAPTER 3. TRANSCRIPTIONAL PROFILING OF MYOSTATIN-KNOCKOUT MICE IMPLICATES WNT SIGNALING IN POSTNATAL SKELETAL MUSCLE GROWTH AND HYPERTROPHY

A summary to be submitted to the FASEB Journal for print publication

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*Department of Animal Science and ‡Department of Statistics, Iowa State University, Ames, Iowa 50011

Specific Aims

The aim of this study was to identify differences in gene expression between myostatin-null and wild-type mice. Elucidation of potential downstream targets of myostatin will increase understanding of the mechanism by which it signals to inhibit skeletal muscle growth.

Principal Findings

1. The number of differentially expressed genes varied significantly across time points

Samples from $Mstn^{-/-}$ and $Mstn^{+/+}$ mice at 2 embryonic and 1 postnatal time point were analyzed using the Affymetrix Mouse Expression Set 430. Allowing for a false discovery rate (FDR) of 0.05, 472 genes were determined to be differentially expressed at 13.5 d.p.c., 73 at 17.5 d.p.c., and 2109 at 35 days of age.

2. Lack of myostatin impacted genes involved in a wide variety of biological processes

Examination of functional annotation by the Expression Analysis Systematic Explorer (EASE) revealed that differentially expressed genes encompassed a range

of categories (**Fig. 1**). At all 3 time points, there were significant changes in metabolism, stimulus response, cell communication, and morphogenesis.

3. Lack of myostatin caused a switch in myogenic fiber type

In pectoralis muscle from 35-day-old mice, significant differences were observed in several muscle-specific genes. In general, the direction of change in myosins, troponins, actins, and other genes supported a shift away from the slow phenotype in the absence of myostatin.

4. Myostatin expression resulted in changes in Wnt pathway signaling

Microarray analysis revealed that a number of genes that encode Wnt pathway components were differentially expressed between $Mstn^{-/-}$ and $Mstn^{+/+}$ mice (**Fig. 2**). Expression changes indicated that the canonical pathway, through β-catenin, was downregulated in the absence of myostatin, while the Wnt/calcium pathway was upregulated.

5. Wnt4 stimulated proliferation of skeletal muscle satellite cells

Satellite cells isolated from $Mstn^{+/+}$ mice were co-cultured with cells producing Wnt4. These cells displayed an increase in proliferation when compared to empty-vector controls.

6. Inhibition of Wnt signaling repressed satellite cell proliferation

Satellite cells obtained from $Mstn^{-/-}$ mice were exposed to cells expressing a combination of the Wnt inhibitors sFRP1 and -2. This resulted in repression of satellite cell proliferation in comparison to empty vector controls.

Conclusions

This study resulted in the identification of over 2000 genes whose expression was affected by a lack of myostatin. A large proportion of the genes were involved in metabolism, with additional changes evident in the categories of cell communication, enzyme activity, morphogenesis, and nucleic acid or protein binding. The multitude of functional categories affected indicate that myostatin is capable of exerting global effects on gene expression.

Because of the exceptional amount of data generated by microarray analysis, we chose to focus further investigation only on the data from 35-day-old mice. At this time point, the gene categories that exhibited changes in expression between $Mstn^{+/+}$ and $Mstn^{-/-}$ mice were consistent with enhanced muscle growth. For instance, effects were observed on genes involved in translation and protein modification, homeostasis, growth, differentiation, and energy production. In addition, several muscle-specific genes were differentially expressed. Genes encoding slow isoforms of several contractile apparatus proteins were downregulated in the presence of myostatin. Since corresponding increases were not observed in genes responsible for fast isoforms, we presume that upregulation of the fast phenotype may occur through changes at the translational or post-translational level. This is supported by EASE analysis, which identified translation as an overrepresented category in the genes upregulated at day 35. These results indicate that myostatin may be capable of evoking a switch in myogenic fiber type, as has been previously reported.

Another notable finding at the day 35 time point was that myostatin genotype appears to have an impact on the expression of a number of Wnt signaling pathway components. Several factors believed to play a role in Wnt/calcium signaling, including Wnt4, were revealed by microarray analysis to be upregulated in $Mstn^{-/-}$ versus $Mstn^{+/+}$ skeletal muscle. In addition, several inhibitors of the canonical Wnt pathway had increased expression. This suggests that Wnt4 signaling may be downregulated in the opposite situation, when myostatin is present.

The results of subsequent experiments point to a role for Wnt4 in skeletal muscle satellite cell proliferation. Exposure to Wnt4 increased proliferation of wild-type satellite cells. Conversely, exposure to Wnt inhibitors, sFRP1 and-2, repressed proliferation of $Mstn^{-/-}$ satellite cells. Although Wnts are known to be capable of inducing embryonic myogenesis, and are able to stimulate proliferation of a variety of cell types, this is the first time Wnt signaling has been implicated in satellite cell proliferation.

In summary, this study identified a multitude of genes that were differentially expressed in the presence or absence of myostatin. Further examination of these genes may provide significant insight into factors that are downstream targets of myostatin, therefore allowing us to better understand the pathway through which it signals. This research also suggests a role for Wnt signaling in postnatal muscle growth and hypertrophy. We propose that myostatin acts upstream of Wnt4, which, in turn, is capable of stimulating satellite cells to proliferate (**Fig. 3**). Taken together, this work should aid in further elucidation of the mechanism whereby myostatin inhibits skeletal muscle growth.

Figure Legends

Figure 1. Biological process annotation of differentially expressed genes. Using DAVID, gene ontology classification was determined for up- or down-regulated genes found to be significant by FDR analysis at 13.5 d.p.c, 17.5 d.p.c, and day 35. At day 35, "other classes" include homeostasis, reproduction, embryonic development, pattern specification, growth, epigenetic regulation of gene expression, and secretion, among others.

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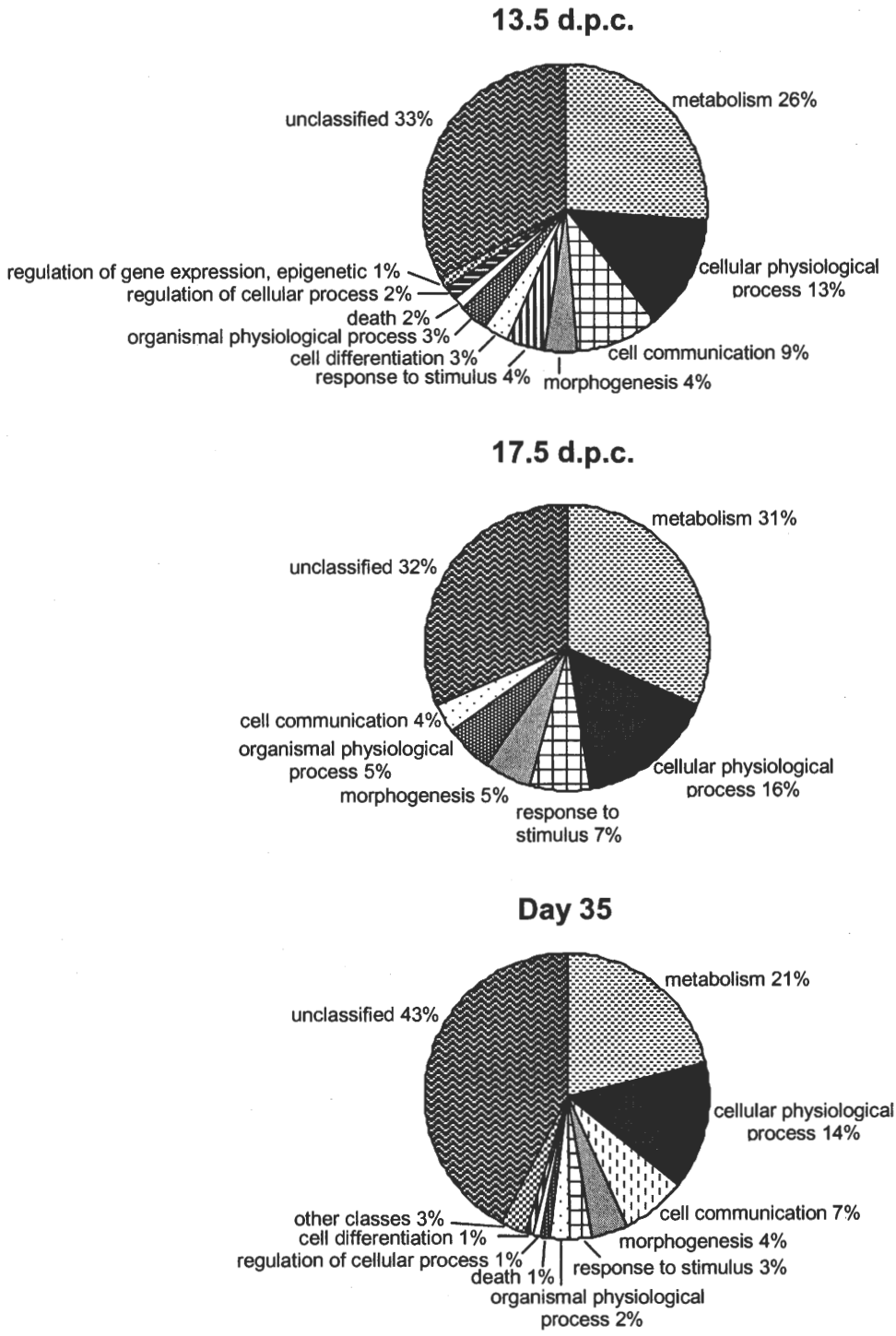
Figure 3. Schematic depicting the proposed role for Wnt signaling in myostatin's repression of skeletal muscle growth.

Figure 1


13.5 d.p.c.
metabolism 26%
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morphogenesis 4%
17.5 d.p.c.
metabolism 31%
unclassified 32%
cell communication 4%
organismal physiological process 5%
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response to stimulus 7%
cellular physiological process 16%
Day 35
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cellular physiological process 14%
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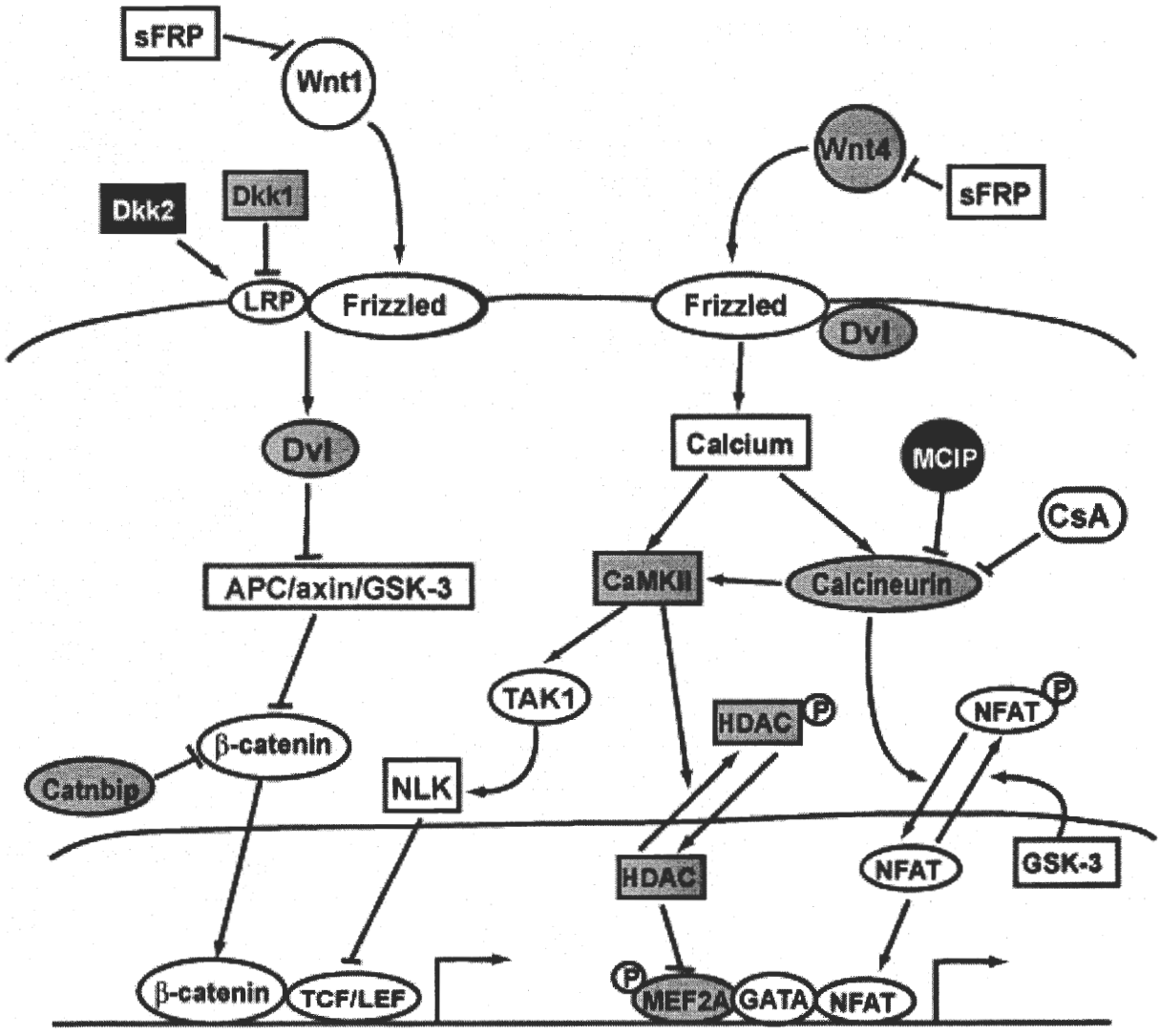
response to stimulus 3%
morphogenesis 4%
cell communication 7%

Figure 2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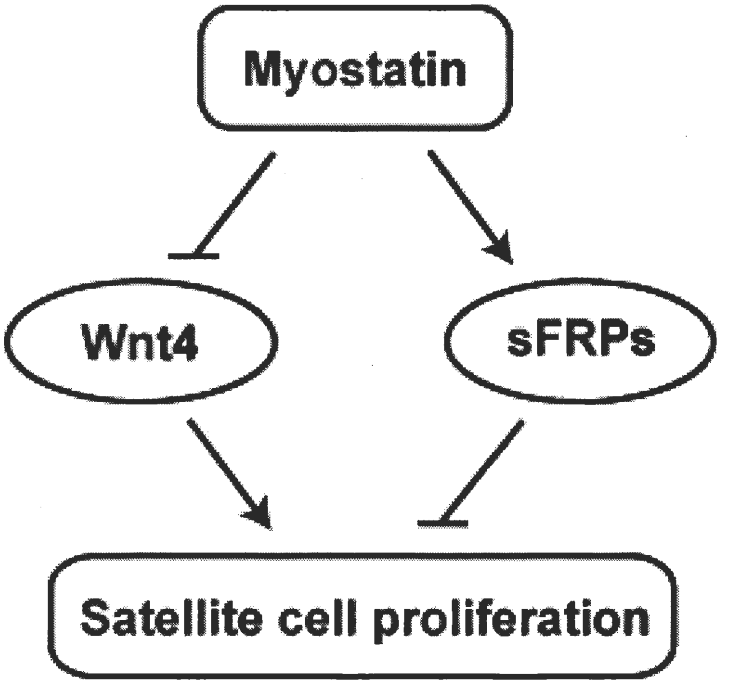
NFAT

Figure 3



CHAPTER 4. GENERAL CONCLUSIONS

The molecular regulation of skeletal muscle growth and hypertrophy is a highly complex process, owing to the involvement of numerous signaling molecules and the potential for crosstalk between pathways. Myostatin's unique ability to act as a specific inhibitor of skeletal muscle growth makes it an attractive target for manipulations intended to increase muscle mass. However, in order to effectively manipulate myostatin signaling, we need to improve our understanding of its mechanism of action.

In this study, expression profiling was used to identify a substantial number of genes and ESTs that were differentially expressed between myostatin-null and wild-type mice. Examination of functional annotation demonstrated that myostatin is able to exert effects on transcription of genes involved in a wide range of biological processes. Only a handful of these genes were subjected to further scrutiny. Closer examination of the remaining genes will quite possibly provide additional insight into factors with which myostatin interacts. In addition, many of the ESTs shown to be differentially expressed remain unannotated. As these become identified, our understanding of myostatin's impact will continue to increase.

This research has implicated, for the first time, a role for Wnt signaling in postnatal muscle growth and hypertrophy, specifically satellite cell proliferation. The results indicate that Wnt4 acts downstream of myostatin, and that when myostatin is not repressing it, Wnt4 can stimulate the proliferation of satellite cells. This finding could be further clarified by examining the effects of *in vivo* Wnt pathway manipulation on satellite cell proliferation. Additionally, expression levels of the

affected Wnt pathway genes could be compared in skeletal muscle from double-muscled and non-double-muscled cattle, to determine if myostatin also affects this pathway in other species.

Future research includes microarray analysis to identify gene expression differences between double-muscle and normal cattle, ideally at time points corresponding to those included in this study. This will provide information on whether the phenotype in cattle results from a similar mechanism as that of the knockout mouse.

In total, this work should prove extremely valuable in enhancing our understanding of the downstream targets of myostatin and the mechanism through which it signals to negatively regulate skeletal muscle growth. The ability to manipulate myostatin pathway components may provide us with novel targets that can be used to combat muscle atrophy.

ACKNOWLEDGEMENTS

First, I'd like to thank my major professor, Jim Reecy. Thank you for challenging me, for your eternal (and sometimes frustrating!) optimism, and for often giving me more credit than I gave myself.

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Finally, I want to thank my boyfriend, Greg, for celebrating my successes and cheering me through the rough times. We made it.