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## Functional profiles of growth-related genes during embryogenesis and postnatal development of chicken and mouse skeletal muscle

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**FUNCTIONAL PROFILES OF GROWTH RELATED GENES DURING  
EMBRYOGENESIS AND POSTNATAL DEVELOPMENT OF  
CHICKEN AND MOUSE SKELETAL MUSCLE**

**HAKAN KOCAMIS**

**Dissertation**

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**the College of Agriculture, Forestry and Consumer Sciences at**

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**in partial fulfillment of the requirements for the Degree of**

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**In Animal and Food Science**

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## ABSTRACT

### Functional Profiles of Growth Related Genes during Embryogenesis and Postnatal Development of Chicken and Mouse Skeletal Muscle

Hakan Kocamis

Myostatin (also known as growth differentiation factor/8), a recently identified member of the TGF- $\beta$  family, has been shown to negatively regulate skeletal muscle growth. Activins, also members of the TGF- $\beta$  family, and their binding protein, follistatin, once thought to be restricted to reproductive cycle function are in fact involved in the development of a wide variety of embryological and adult tissues, particularly skeletal muscles. Reverse-transcription polymerase chain reaction (RT-PCR) was performed to measure the ontogeny of myostatin, activin-B, and follistatin gene expression during chicken embryonic development. Strong myostatin expression was found in the early chicken embryos (E 0, E 1) and the developmental expression pattern of myostatin mRNA coincided with the periods of primary and secondary muscle fiber formation. Follistatin transcripts followed a linear expression pattern from E 0 to E 20, while activin-B had a quadratic pattern.

The ontogeny of myostatin gene expression was nearly identical in satellite cells isolated from *pectoralis major* (PM) and *biceps femoris* (BF) muscles of chicken. Activin-B mRNA level in PM satellite cells was higher than in BF satellite cells at 72 h and 120 h ( $P < 0.01$ ), whereas levels in BF satellite cells were higher than in PM satellite cells at 96 h and 144 h ( $P < 0.01$ ). Amounts of follistatin mRNA in PM satellite cells were higher than in BF satellite cells at 24, 96, and 120 h of culture ( $P < 0.01$ ). No IGF-I gene expression was detected in either cell culture at any time point in the present study. IGF-II mRNA level plateaued in PM satellite cells by 48 h after plating ( $P < 0.05$ ), and remained elevated until 144 h of culture. *In ovo* administration of rhIGF-I at E 3 altered myostatin, follistatin, activin-B, and TGF- $\beta$ 2 gene expressions during chicken embryonic development with emphasis on skeletal muscle development. Myostatin mRNA from pectoralis muscles of rhIGF-I injected embryos increased on E 10 (~ 2.5 fold) and remained high through E 13, whereas mRNA from control pectoralis muscles increased at E 9 and remained high until E 12.

IGF-I, -II and IGF receptor-I mRNA and protein levels were determined in a wide variety of myostatin knockout mice tissues. IGF-I mRNA levels were not different between control and knockout mice tissues, whereas levels for IGF-II were significantly higher in myostatin knockout mice kidney and soleus muscles than that of control mice ( $P < 0.01$ ). IGF-Receptor-1 mRNA levels from control mice heart ( $P < 0.05$ ) and kidney ( $P < 0.01$ ) were significantly higher than that of myostatin knockout mice, while levels were lower in control mice pectoralis muscle than that of knockout mice ( $P < 0.01$ ). The strongly IGF-II positive cells were more common in myostatin knockout mice and were seen in a few foci in control mice, while no consistent differences in IGF-II immunoreactivity were detected between the two groups of mice kidneys.

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## INTRODUCTION

A series of defined cellular events orchestrates the development of skeletal muscle. First, mesodermal cells become epithelized and partitioned into the somites (Christ et al., 1983). Dorsal parts of the somites form the dermomyotome, which ultimately gives rise to the dermis and skeletal muscle of the back, body wall and limbs (Ordahl and Le Douarin, 1992). Then, myoblasts undergo terminal differentiation to myocytes expressing contractile proteins characteristic of skeletal muscle (Cusella-De Angelis et al., 1992). Finally, myocytes align and fuse to form multinucleated myotubes. Sequential myofiber formation establishes the muscle groups. Primary myofibers, which form from the first wave of myoblasts, are the first to arrive at the pre-muscle mass, while secondary fibers, which are late migrating myoblasts, develop around the primary fibers. It has been suggested in chickens that the myoblast withdrawal process is biphasic, starting at embryonic day (E) 7 (primary myofibers), then stopping, and starting once again around E 11 (secondary myofibers) (O'Neill, 1987). The ratio of terminally differentiated myocytes in the embryonic breast and thigh muscles increases from a few percent at E 7 to 80% at E 18 (O'Neill, 1987). The schematic presentation of key myogenic events during chicken embryonic development is provided in the Appendix of this dissertation.

The precise mechanisms that control the events converting somites to functional muscle remain largely unknown. Nevertheless, based on recent *in vitro* findings and advances in developmental biology techniques, it has been suggested that growth factors such as, insulin-like growth factors (IGFs), transforming growth factor betas (TGF- $\beta$ s),

and fibroblast growth factors (FGFs) play a significant role in coordinating these processes. Particularly, myostatin (also known as growth differentiation factor/8), a recently identified member of the TGF- $\beta$  family, has led scientists to reevaluate their concepts about muscle development in vertebrates. Based on the observations obtained from myostatin knockout mice that displayed a marked increase in muscle mass, up to three times normal size (McPherron et al., 1997), myostatin has been proposed to be a negative regulator of skeletal muscle growth. Additionally, activins, also members of the TGF- $\beta$  family, and their binding protein, follistatin, once thought to be restricted to reproductive cycle function are in fact involved in the development of a wide variety of embryological and adult tissues, particularly skeletal muscles. Therefore, myostatin, activin, and follistatin expression and their biological functions are reviewed in the following section.

## **LITERATURE REVIEW**

### **MYOSTATIN**

How is the size of an animal determined? Most importantly, how is the growth of an individual tissue controlled to reach and maintain its proper size? These questions remain as mysterious as ever.

There has to be a mechanism to coordinate the growth of each tissue so that they can produce harmony in the whole organism. Several theories have been suggested to explain this phenomenon. The most favored by scientists in the field of growth biology is that each tissue produces an inhibitor that specifically suppresses the growth of itself (Bullough, 1962). As the organ or tissue grows, the inhibitory substance accumulates until it reaches a threshold causing cessation of growth of the tissue or organ from which

it was produced (Goss, 1978). This theory can relate to liver regeneration as described as early as in ancient Greek mythology (for review, see McPherron and Lee, 1999) and in grafting experiments. For instance, the removal of part of the liver causes rapid compensatory growth of the remainder. Also, when a small liver is transplanted to a large host, it will grow much faster than the normal rate until it has reached proportional size of the host (Kam et al., 1987). Because of the lack of direct evidence for tissue specific inhibitory molecules, this once-widely-accepted theory fell out of favor. However, based upon recent findings, this type of mechanism can operate in skeletal muscle growth. When myostatin (also known as growth and differentiation factor/8), a member of TGF- $\beta$  family, was disrupted in mice, muscle mass increased up to three times normal size (McPherron et al., 1997). Additionally, myostatin mutation has been linked to double muscled cattle breeds (Grobert et al., 1997, Kambadur et al., 1997). It is, therefore, suggested that myostatin is the negative regulator of muscle growth in normal animals.

### ***Myostatin Structure and Expression***

Myostatin is mainly synthesized in skeletal muscle as a 376 a.a. propeptide, which gives rise to 15 kDa active, processed and mature protein (McPherron et al., 1997). Structurally, it contains all the characteristic features of the TGF- $\beta$  family, such as a proteolytic processing signal site and an active carboxy-terminal region that has the highly conserved patterns of cysteine knots (McPherron et al., 1997). The a.a. sequence of the active, proteolytically-processed carboxy-terminal site of myostatin has 100% homology among murine, rat, human, porcine, chicken, and turkey species (McPherron and Lee, 1997), which suggests a common and highly conserved function.

Myostatin was detected very early in the myotome of developing mouse (McPherron et al., 1997) and cattle embryos (Kambadur et al., 1997) and expression continued in the adult muscle. It was located in the cytoplasm of muscle fibers and was absent from connective tissue (Kirk et al., 2000). Myostatin protein level was higher in the slow-fiber-type dominated muscles (soleus) than in the fast-fiber-type dominated muscles (tibialis anterior) of rats (Sakuma et al., 2000). On the contrary, myostatin mRNA and protein level were higher in fast-fiber-dominated muscles (gastrocnemius/plantaris) than in slow-fiber-dominated muscles (soleus) of mice (Carlson et al., 1999) and rats (Wehling et al., 2000). In humans, there was no difference between fast-fiber-dominated and slow-fiber-dominated muscles in terms of their myostatin gene expression (Gonzalez-Cadavid et al., 1998). In chicken satellite cells isolated from pectoralis major (predominantly fast fibers) and biceps femoris (predominantly slow fibers) muscles, myostatin mRNA expression was nearly identical (Fig. 1) with the exception of significant increase when fusion started in biceps femoris satellite cells (Kocamis et al., 2001). Despite the various results among the species, myostatin may be one of the major determinants of muscle fiber type in any given muscle. On the other hand, myostatin expression is not certainly limited to skeletal muscle tissues. For instance, its expression was detected in cardiomyocytes and purkinje fibers of cattle heart (Sharma et al., 1999), and in adipose tissue and tubuloalveolar secretory lobules of lactating mammary glands in pigs (Ji et al., 1998).

### ***How Myostatin Functions***

Myostatin knockout mice demonstrated a dramatic and widespread increase in skeletal muscle mass due mainly to increase in the number of muscle fibers (hyperplasia)

and also the thickness of the fibers (hypertrophy), irrespective of age and sex of animals (McPherron et al., 1997). In addition, some of the double muscled cattle breeds (discussed below) have been found to possess loss of function mutations in the myostatin coding sequence (Kambadur et al., 1997, Grobert et al., 1997).

It is well known that the size of any given tissue depends on the number and size of the cells it contains as well as on the amount of extracellular matrix and fluid (for review, see Conlon and Raff, 1999). Cell division and cell death determine appropriate cell number in a tissue at any time during development. Also, both cell number and cell size in a tissue depend on interaction between intracellular programming and extracellular signaling. Currently, it is not known whether myostatin is present in circulation of any vertebrate. Thus, we will ignore any possible endocrine functions of myostatin. To increase hyperplastic myoblast growth, myostatin must be acting either by interrupting normal myoblast cell cycles through changing the activity and/or amount of regulatory enzymes and/or other components, or by preventing apoptosis. *In vitro* studies demonstrated that recombinant myostatin inhibited the proliferation of C2C12 myoblasts and bovine myoblasts derived from 160-day-old fetuses (Thomas et al., 2000). It was shown that the inhibitory effect of myostatin was reversible, as myoblasts maintained their ability to proliferate after it was removed. Extra myostatin specifically upregulated P<sup>21</sup>, a cyclin-dependent kinase inhibitor, and decreased the amount of cyclin-dependent kinases (especially cdk2), a family of enzymes that catalyze events required for cell cycle transition, in C2C12 cells (Thomas et al., 2000, Rios et al., 2001). Both groups suggested that myostatin blocked the myoblast transition in the G1/S and/or G2/M phases of the cell cycle. On the other hand, they found conflicting results in terms of myostatin effects on

apoptosis, even though they used the same myoblast cell culture. For instance, Thomas et al. (2000) demonstrated that myostatin did not affect apoptosis in C2C12 cells as shown in TUNEL assays, whereas Rios et al. (2001) showed that myostatin overexpression inhibited apoptosis in the same cells. Therefore, further studies should be conducted to better understand myostatin involvement in apoptosis.

Myostatin dominant negative mice expressing 23-40% less active myostatin due to the lack of its normal cleavage site, showed widespread hypertrophic muscle growth but not hyperplasia (Zhu et al., 2000). Muscle hypertrophy in these mice was not due to the pathways that involve well established transcription factors such as myogenin, GATA-2, and MEF-2C. Because myostatin knockout mice (fully null for myostatin) demonstrated both hyperplastic and hypertrophic muscle growth, it is possible to postulate that the hypertrophic function of myostatin is independent of its hyperplastic functions. On the other hand, less inhibition of myostatin may be sufficient for hypertrophy, whereas complete or greater inhibition may be required for hyperplasia.

Experiments with muscle atrophy and mechanically-induced hypertrophy were conducted to address the possible myostatin function in hypertrophy of fibers of fully differentiated muscle. Muscle atrophy caused by either hindlimb unloading (Wehling et al., 2000, Carlson et al., 1999) or by denervation (Sakuma et al., 2000) increased myostatin mRNA and protein level. These observations were supported by findings of Gonzalez-Cadavid et al.(1998) who demonstrated that myostatin protein level increased in the muscle of HIV-infected patients undergoing weight loss. On the contrary, myostatin protein level was also increased in mechanically-hypertrophied rat muscles. As widely accepted, mechanical overloading does not induce new fiber formation (Gollnick



et al., 1981), but appears to promote the hypertrophy of originally present muscle fibers by stimulating DNA synthesis. However, recombinant myostatin inhibited DNA and protein synthesis without affecting protein degradation in C2C12 cells (Taylor et al., 2001) and also myostatin mRNA level was not altered in hypertrophied C2C12 myotubes transfected with IGF-I (Semsarian et al., 1999). The exact mechanism of myostatin function in hypertrophy, thus, remains to be elucidated in further studies.

Hypertrophic growth is associated with satellite cell replication in order to maintain constant nuclear number (Snow, 1990). However, little attention has been paid to involvement of myostatin in the process of satellite cell proliferation and differentiation. Myostatin mRNA was detected in satellite cells isolated from chicken pectoralis major and biceps femoris muscles during proliferation (Fig. 1, Kocamis et al., 2001). Also, it increased during differentiation of either satellite cell culture, thus myostatin may be one of the major determinants of satellite cell activation to maintain overall postnatal muscle mass in vertebrates.

### ***Myostatin in Double Muscling***

Double muscling, also known as muscle hypertrophy (*mh*), is a phenotype recognized as early as the 19<sup>th</sup> century, characterized by a visible, generalized increase in muscle mass due primarily to hyperplasia and is present in Belgian Blue, Piedmontese and Asturiana de los Valles cattle breeds. These animals have higher birth weights and also lower fat and bone percentages compared to non-double muscled animals (for review, see Arthur, 1995). Comparative mapping analysis between human and bovine genomes led to identification of the *mh* locus underlying double muscling (Charlier et al., 1995). However, none of the genes identified within this locus was considered to be a

strong candidate for the double muscle phenotype, until the study of myostatin knockout mice was published (McPherron et al., 1997). Location of the myostatin gene was found to be in *mh* locus of cattle chromosome 2 by mapping with genetic markers (Smith et al., 1997, Casas et al., 1999). Given the phenotype of the myostatin knockout mice and location of myostatin on cattle chromosome 2, the question was if myostatin was the *mh* gene. Disruption in the timing, distribution, level of expression or function of the protein should be the cause of double muscle phenotype in cattle. Because no difference was found between double muscled and normally muscled cattle embryos or their adult muscles in terms of timing or level of expression (Kambadur et al., 1997, Grobert et al., 1997), mutations at the protein level were likely responsible for the phenotype in these breeds. Indeed, three independent studies, almost concurrently, demonstrated that double muscled Belgian Blue, Piedmontese and Asturiana de los Valles breeds had mutations in their myostatin genes (Grobert et al., 1997, Kambadur et al., 1997, McPherron and Lee, 1997). For instance, Belgian Blue cattle have an 11 base pair deletion in the myostatin gene, resulting in a translational frame shift which causes functional loss. The South Devon cattle breed has the same mutation in their myostatin gene, but they are not considered as true double muscled animals (Smith et al., 2000). Additionally, Limousin and Blonde D'Aquitaine cattle breeds do not demonstrate an observed double muscling phenotype, although a functional myostatin mutation was found in these animals (Grobert et al., 1998). Based upon all of these results, the function of single major gene may not completely explain the double muscling phenotype.

The negative aspects of double muscled phenotypes are calving difficulties, and smaller internal organs (Arthur, 1995). Interestingly, cattle with only one functional copy

of the myostatin gene (+/-) had a significant increase in muscle growth, while having a lower incidence of calving difficulties than homozygous animals (-/-) (Casas et al., 1999). These findings sharply contradict with the situation in mice, which show little difference between wild type (+/+) and heterozygous (+/-) animals in muscle weight (McPherron et al., 1997). Cattle, because of extensive selection for large muscle mass, unlike mice, may have associated genetic changes that allow for increase in muscling in a heterozygous animal. Genetic background may be critical in determining the phenotypic response to myostatin mutations.

Leg weakness (such as twisted leg, chondrodystrophy, and tibial dyschondroplasia) continues to be a serious problem among meat-type poultry, particularly with selection for increased body weight gain (Cook et al, 1984). Despite the extensive muscle growth of the myostatin knockout mouse, its femora were not altered in either shape or size (Hamrick et al., 2000). It is imperative to note that body weight of the myostatin knockout mouse was identical to the wild-type control, while it had extreme muscle growth. Thus, ablation of myostatin during chicken embryonic development may prevent chickens from having weight-associated skeletal abnormalities, while increasing muscle mass.

Although the phenotype of myostatin deficient animals allows the possibility that myostatin may be the specific growth inhibitor that was speculated in the early 1960's (Bullough, 1962), several questions remain to be elucidated in terms of exact mechanisms of myostatin function. First, does myostatin circulate in the blood of any given animal? If so, do binding proteins in the TGF- $\beta$  family such as follistatin and noggin regulate its activity in circulation? Second, does myostatin have a specific receptor or does it use a

common TGF- $\beta$  family receptor? Third, can myostatin inhibit the growth of skeletal muscle in adult animals? If so, is it dose-dependent? Fourth, is myostatin involved in highly muscled callipyge sheep (Cockett et al., 1994) or Pietrain pigs (Brenig and Brem, 1992)? Fifth, how does myostatin interact with the growth factors that have been well documented to stimulate skeletal muscle growth, such as insulin-like growth factors (IGF-I and IGF-II)? Therefore, complete understanding of the biochemistry and physiology of myostatin could be beneficial to human health and food animal agriculture.

## **ACTIVINS and FOLLISTATIN**

### ***Activins Structure and Expression***

Activins were first found in 1986 as a novel protein purified from ovarian follicular fluid (Vale et al., 1986). They are categorized as members of the TGF- $\beta$  family and are present as a dimeric polypeptide linked by one disulfide bond between residue 80 of the mature subunits (Vale et al., 1986). Activins are comprised of homodimers ( $\beta$ A $\beta$ A or  $\beta$ B  $\beta$ B) or heterodimers ( $\beta$ A $\beta$ B), resulting in activin-A, -B and -AB, respectively (for further review, see DePaolo, 1997). Recently, three more activin subunits ( $\beta$ C,  $\beta$ D,  $\beta$ E) were found in humans but their functions remain unknown (Hotten et al., 1995, Fang et al., 1996). Inhibins, which are structurally related to activins, are also dimeric proteins comprised of an  $\alpha$  subunit and one of the two  $\beta$  subunits ( $\beta$ A or  $\beta$ B), thus existing in two forms  $\alpha\beta$ A or  $\alpha\beta$ B. Southern blot analysis has shown that activin subunits are encoded by different genes and that they are present as single copies in the human genome (Thompson et al., 1994, Feng et al., 1989). Additionally, these subunits are very conserved among the species in terms of genetic organization (Thompson et al., 1994, Feng et al., 1989).

Activins are ubiquitously expressed in a wide variety of embryonic and adult tissues. For instance, they were detected in human as well as rat embryo and adult nervous system, bone marrow, reproductive tissues, spleen, liver, heart, muscle, and salivary glands (Ying et al., 1987). Activin mRNAs were also uniformly distributed at the blastula and gastrula stages of xenopus embryos (Dohrmann et al., 1993). In the chicken, activin transcripts were first detected during the period of axial mesoderm formation (Mitrani et al., 1990).

### ***Follistatin Structure and Expression***

Follistatin is present as a monomeric polypeptide subject to post-translational modification resulting in different molecular weights of bioactive proteins (ranging from 31 to 42 kDa) (Robertson et al., 1987). Sequence analysis demonstrated that the follistatin gene consists of six exons separated by five introns (Shimasaki et al., 1988). This single follistatin gene is subject to alternative splicing, which in turn gives rise to follistatin 288 (FS 288) and follistatin 315 (FS 315) in humans (Inouye et al., 1991). However, analysis of native porcine follistatin failed to detect intact FS 315.

Follistatin is broadly distributed in embryonic and adult tissues and is not confined to reproductive tissues. It is also present in the circulatory system and binds to activin via their  $\beta$  subunits (Patel, 1998). Follistatin was first seen during gastrula stages of xenopus embryos (Matzuk et al., 1995). Follistatin-activin coexpression in developing mouse kidneys, salivary glands, liver, heart and skeletal muscle was observed. Additionally, follistatin demonstrated an expression pattern parallel to that of activins, implying that the activin-follistatin system may act locally and at multiple sites during early development (Matzuk et al., 1995).

In chickens, follistatin was first detected in the cranial segmental plate mesoderm and subsequently in all somites (Amphor et al., 1996). Within somites, its expression was localized to the dorso-lateral part of the somites, which give rise to skeletal muscle of body walls, and limbs (Christ, 1977, Ordahl and Le Dourarin, 1992). Then, expression of follistatin was detected in myotomes and migrating myogenic cells (Amphor et al., 1996). Taken together, follistatin gene expression in chicken somites follows a pattern suggestive of a role in regulating muscle development (Connolly et al., 1995).

### ***Activin Receptors***

The presence of activin membrane surface receptors was first reported in the late 1980s (Sugino et al., 1988). Since then, four types of activin receptors (type-I, -IB, -II and -IIB) have been identified (Ying et al., 1997). Although they are ubiquitously expressed in a wide variety of embryonic and adult tissues, some isoforms have been found to be expressed only in embryos and certain cell types. For instance, type-II activin receptors were expressed in the embryonic pituitary glands of rats, while only activin receptor type-IIB was found to be expressed in the adults (Roberts and Barth, 1994).

Activins bind to constitutively activate type-II serine/threonine receptors, but can not interact with the intracellular signaling components of pathways without recruiting type-I receptors to the activin-type-II complex (Wrana et al., 1994). Type-I receptors, unlike type-II receptors, do not bind activins without the presence of type-II receptors (Ebner et al., 1993, Attisano et al., 1993). Therefore, activins must first bind to type-II receptors, thereafter type-I receptors are recruited to the complex and become phosphorylated, then this heteromeric complex initiates intracellular signal transduction pathways. To form this heteromeric complex, activin binds to each receptor through its  $\beta$

subunits (Xu et al., 1995). Thus, when inhibins that have the same  $\beta$  subunits as activins, bind to type-II receptors, they can not assemble the heteromeric complex due to the lack of a second  $\beta$  subunit in their structure. Additionally, even though activin type-II receptors are structurally similar to the TGF- $\beta$  type-II receptors, TGF- $\beta$ s do not bind to these activin receptors (De Winter et al., 1996).

It was recently demonstrated that some of the xenopus and zebrafish embryonic and adult tissues contain a pseudoreceptor named BAMBI (BMP and activin membrane-bound inhibitor) (Onichtchouk et al., 1999). It has a type-I receptor-like structure but lacks a full intracellular domain and thus, can not be phosphorylated. However, BAMBI can compete with full-length activin type-I receptor to form a heteromeric complex with activin ligand, thereby inhibiting the potential for signal transduction. Given the fact that activins have multiple effects on a variety of reproductive and non-reproductive tissues and that the amount of ligand required for proper response varies among tissues during development (for details see below), many isoforms of activin receptors and the presence of pseudoreceptors should be necessary to maintain desired physiological responses.

### ***Biological Functions of Activins and Follistatin***

Activins have been proposed as potential local hormones in the regulation of gonadal cell growth and differentiation as well as follicle stimulating hormone (FSH) synthesis and secretion. Therefore, the majority of activin and follistatin studies have been focused primarily on reproductive tissues. For instance, in the absence of follistatin, activins stimulated the synthesis and secretion of FSH, while simultaneously suppressing the secretion of growth hormone, prolactin, and adrenocorticotrophic hormone (ACTH), thereby enabling the organism to enter the reproductive phase. On the contrary, in the

presence of follistatin, activins were not able to allow the organism to enter the reproductive phase. Because it is not the intention of this review to cover all of the biological functions of activins and follistatin in reproductive tissues, two excellent reviews are recommended (Knight, 1996, DePaolo, 1997).

The phenotype of subunit knockout mice has demonstrated that each activin has its own function on different tissues. For instance,  $\beta$ A subunit knockout mice lacked whiskers and lower incisors and had cleft palates, while  $\beta$ B subunit knockout mice showed distinct developmental and reproductive defects as well as severe eye lesions (Vassalli et al., 1994). Double knockout mice for  $\beta$ A and  $\beta$ B demonstrated individual defects of the subunits without any additional defects (Matzuk et al., 1995), implying that the absence of one subunit is not compensated by another subunit. Follistatin knockout mice were much smaller than their heterozygous litter mates and had declined mass of diaphragm, pectoral, and intercostal muscles (Matzuk et al., 1995). These mice failed to breathe and died soon after birth. They also displayed some skeletal abnormalities as well as defects of their teeth and whisker development. Follistatin knockout mice demonstrated a much wider range of improper development than activin knockout mice, indicating that follistatin may function in a wide range of tissues and that it may also be involved in the regulation of other TGF- $\beta$  family members. Additionally, follistatin transgenic mice did not die at birth and were of normal size (Guo et al., 1998). However, because males had smaller testes and females had smaller ovaries and uteri, their fertility rates were reduced.

Recently, many studies have demonstrated that activins and follistatin play a pivotal role in the induction of mesoderm and neural tissues of embryos. For example, in



xenopus embryos, addition of extra activins stimulated mesodermal tissue formation (Thomsen et al., 1990). At high dosage, embryos even displayed a rudimentary axial pattern and head structure. Furthermore, a dominant negative receptor for activins blocked the mesoderm formation in these embryos (Hemmati-Brivanlou et al., 1992). Based on these findings, it is possible to postulate that endogenous activins regulate mesodermal patterning. On the other hand, overexpression of follistatin in early xenopus embryos did not block mesoderm induction, while inducing neural tissue formation (Sculte-Merker et al., 1994).

Activins induced cardiac myogenesis in *in vitro* chicken embryonic epiblasts (stage 11 or 15, embryonal days 2 and 2.5 day, respectively) (Ladd et al., 1998). Addition of follistatin in the medium of these cultures inhibited activin induced cardiac myogenesis. Furthermore, recombinant human activin A inhibited differentiation in 11 day-old chicken embryonic pectoralis muscles in culture, while recombinant human follistatin stimulated muscle cell differentiation (Link and Nishi, 1997). Also, myotubes that were formed in the presence of activin had fewer nuclei and lacked parallel alignment. On the other hand, cultures grown with follistatin had thicker myotubes that were aligned in parallel fashion. The inhibitory action of activin on those myoblasts was distinct from FGFs and TGF- $\beta$ s (Link and Nishi, 1997). For instance, either TGF- $\beta$  or FGF delayed the onset of muscle differentiation, but unlike activin treated cultures, these cultures eventually reached the control creatine kinase levels, indicative of myotube maturation. Additionally, activin treated cultures had consistently lower myoD and myf5 mRNA levels than untreated controls. However, follistatin treated cultures had a decrease in myoD levels and an increase or no change in myf5 levels compared to

controls, indicating that the actions of follistatin are not simply reciprocal to those of activins.

The aforementioned *in vivo* and *in vitro* functions of activins and follistatins leads to the question of how follistatin and activin interact or regulate each other's action at either the cellular or endocrine levels. Because both activins and follistatins are present in circulation, it is assumed that they function as classic endocrine regulators. At the cellular level, two theories have been suggested. The first is that follistatin captures activins and sequesters them in the cell matrix as a reservoir for future signaling, so that follistatin would prevent rapid clearance of activins (Nakamura et al., 1991). Second, and the most acceptable theory, is that follistatin bound to the cell surface allows degradation of activins by internalizing the activin and follistatin complex into the cell, so that follistatin actually facilitates clearance of activins (Hashimoto et al, 1997).

Because no follistatin specific receptor has been found, follistatin has been shown to have a high affinity for cell surface proteoglycans (particularly heparin sulfates). One of the major roles of these proteoglycans is to immobilize or sequester growth factors, so that their actions can be facilitated (Ruoslahti and Tamaguchi, 1995). Although the affinities of two major forms of follistatin (FS 315, FS 288) for activins are almost identical (Mathews and Vale, 1991), the FS 288 form of follistatin has greater affinity for heparin sulfate proteoglycans than FS 315 form (Sugino et al., 1993). Therefore, the greater effect of FS 288 to suppress FSH secretion has been attributed to greater affinity of this follistatin form for heparin sulfates.

Given the fact that their multifactorial control mechanism, it is difficult to pin point what the exact nature of follistatin-activin interaction is in non-reproductive tissues

such as skeletal muscles. For example, how does follistatin interact with other well known growth factors such as IGFs in skeletal muscle development? Also, do activins and follistatin function in the same fashion among several different non-reproductive tissues such as bone, adipose tissues?

**Chapter 1.**

**THE ONTOGENY OF MYOSTATIN, FOLLISTATIN AND ACTIVIN-B MRNA  
EXPRESSION DURING CHICKEN EMBRYONIC DEVELOPMENT**

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## INTRODUCTION

Myostatin is a member of the transforming growth factor- $\beta$  superfamily (TGF- $\beta$ ) (McPherron et al., 1997). The main source of myostatin synthesis and secretion is skeletal muscle tissue (McPherron et al., 1997). Myostatin null mutation mice showed a dramatic increase in skeletal muscle mass, primarily due to an increased number of muscle fibers (McPherron et al., 1997, McPherron and Lee, 1997). The “double muscle” phenotype of two breeds of cattle (Belgium Blue and Piedmontese) also has been linked to five different myostatin gene mutations (Grobert et al., 1998); however, the developmental pattern of myostatin gene expression in chicken embryos has not been elucidated. Therefore, one of the objectives was to establish the ontogeny of myostatin gene expression during chicken embryonic development, with emphasis on skeletal muscle development.

Activins which are members of the TGF- $\beta$  superfamily are covalently linked dimers of two distinct  $\beta$  subunits, thus existing in three different forms, activin-A, AB, and B (Ying, 1987). Activins and their receptors (type-I and type-II serine/threonine kinase) are ubiquitously expressed (Tuuri et al., 1994). Activins have multiple biological effects in a wide variety of reproductive and non-reproductive tissues. Follistatin is a monomeric glycosylated protein present in several isoforms (for reviews, see Refs. Michel et al., 1993, Ying et al., 1997, Patel, 1998) and is also expressed ubiquitously (Tuuri et al., 1994). Most, if not all, of the biological actions of activins are neutralized by its binding to follistatin (Michel et al., 1993). Furthermore, follistatin binds to inhibins with less affinity, but the physiological relevance of biological activity of inhibin bound

to follistatin has not been fully explained. Additionally, the antagonistic actions of follistatin may extend to other TGF- $\beta$  family members such as, bone morphogenic proteins (BMP) (Amphor et al., 1996).

The expression and regulation of follistatin in the somite and hindbrain of early chick embryos have been reported (Amphor et al., 1996, Graham and Lumsden, 1996). Furthermore, the follistatin gene in early somites is expressed in a way which suggests regulation of skeletal muscle development (Amphor et al., 1996). Beyond these developmental stages mentioned above, follistatin and activins gene expression as related to skeletal muscle development in the chicken has not been fully elucidated. Therefore, the present study expands on the previously published studies by examining the expression of the follistatin and activin-B gene as well as myostatin in the whole embryo and *pectoralis* muscle of the developing chicken embryo.

## **MATERIALS AND METHODS**

### **Tissue collection**

Fertilized eggs (Cobb X Cobb) were obtained from Wampler-Longacre (Moorefield, WV). Embryos and tissues were harvested in compliance with an approved West Virginia University Animal Care and Use Committee Protocol. All the embryos were isolated and washed free of yolk, albumen and extra-embryonic membranes by sterile nuclease-free water and were staged according to Hamburger and Hamilton, (1951). Whole embryos were collected on each of the embryonic days (E) 0 to 6 (starting stage 1 to stage 29, n= 6 per day). Thoracic/abdominal halves of the embryos between lumbo-sacral level to neck without head were collected on each of E 7 and E 8 (stage 31 and stage 34, respectively, n= 6 per day). *Pectoralis* muscle was collected on each of E 9

to E 20 (n= 4 per day). All the tissue collections were performed at consistent times for each sampling day throughout the experimental period, starting day 9, stage 35, and every 24 h until day 20, stage 45 (Hamburger and Hamilton, 1951).

### **RNA extraction**

Total RNA was extracted from all of the tissues mentioned above using the Tri-Reagent (Sigma) modification of the guanidine isothiocyanate/phenol-chloroform method as described by Chomczynski and Sacci (1987). The RNA concentration was estimated by absorbance at 260 nm in a Shimadzu spectrophotometer (Columbia, MD). Samples of RNA were stored at -80 C.

### **RT-PCR**

Reverse transcription (RT) was performed by adding 2 µg of total RNA to 2 µg of Oligo dT primers and sterilized nuclease-free dd H<sub>2</sub>O in a final volume of 15 µl. The samples were heated at 70 C for 5 minutes and then immediately cooled to 4 C for 2 minutes. Reverse transcription buffer containing dNTPs (final concentration of each was 10 mM), 25 units of RNase inhibitor, and 200 units of murine maloney leukemia virus reverse transcriptase (Promega, Madison, WI) were added to each sample. The sample, with a final volume of 40 µl, was incubated at 37 C for 1 hr followed by a 5 minutes incubation at 95 C. For the PCR reaction, 2 µl of RT reaction mixture were added to 48 µl of solution containing 5 µl of Taq buffer, 1 µl Taq DNA polymerase (Display Systems Biotech, Vista, CA), 1 µl dNTPs (final concentration of each was 10 mM), 1 µl each of forward and reverse primers and 41 µl sterile nuclease-free dd H<sub>2</sub>O. The PCR reaction started with one cycle consisting of 94 C for 5 minutes, an annealing step of 65 C for myostatin and activin-B (55 C for follistatin and β-actin) for 1 minute and extension at 72

C for 10 minutes. The first cycle was followed by 30 cycles (25 cycles for  $\beta$ -actin) consisting of 30-sec intervals of 94 C, followed by 65 C for myostatin and activin-B, and 55 C for follistatin and  $\beta$ -actin, followed by 72 C. To establish a linear range of amplification for each gene, several different cycle numbers of PCR (10, 15, 20, 25, 30 and 35 cycles) were run. As a control, PCR reaction mixture without c-DNA was run and found no contamination in reaction mixture (data not shown).

### **PCR primers**

All PCR primers were made by Gibco BRL Inc. (Grand Island, NY). Primers for myostatin were designed on the basis of published sequences of chicken myostatin (McPherron et al., 1997). The sequence of the forward primer was 5' GACTATCATGCCACAACCGAGACGA 3', while the reverse primer was 5' GTGTACCAGGTGAGTGTGCGGGTATT 3'. Forward and reverse primers predicted a PCR product of 657 base pairs (bp), which corresponds to bases 327-984 of the sequence. Primers for follistatin were designed on the basis of the published sequence of chicken follistatin (Graham and Lumsden, 1996). The sequence of the forward primer was 5' CATCCCGTGCAAAGAAAC 3', while the reverse primer was 5'CTCGTAGGCTAATCCAATG 3'. These primers amplified a PCR product of 445 bp as previously reported (Davis and Johnson, 1998), which corresponded to bases 260-705 of the sequence. Primers for activin-B were based on a published partial sequence (Mitrani et al., 1990). The sequence of the forward primer was 5' TACTGTGAAGGGAGCTGCCCCG 3', while the reverse primer was 5' GTACAGCATTGACATTGTGC 3'. These primers amplified a PCR product of 162 bp as previously reported (Davis and Johnson, 1998), which corresponded to bases 13-175



of the sequence. Primers for  $\beta$ -actin were used to amplify a 285 bp product as previously published (Yamamura et al., 1991), as an internal standard to verify the level of amplification. The sequence of the forward primer was 5' TCATGAAGTGTGACGTTGACATCCGT 3', while the reverse primer was 5' CCTAGAAGCATTGCGGTGCACGATG 3'.

The amplified PCR products for each gene were visualized on 1.5 % agarose gels stained with ethidium bromide. Products were quantified by densitometric analysis of stained gels. The identity of all PCR products was confirmed by sequence analysis.

### **Statistical analysis**

Regression analysis was performed by the GLM procedure of SAS<sup>®</sup> (1989). Statements of significance were based on  $P < 0.05$  unless otherwise noted.

## **RESULTS AND DISCUSSION**

### **Myostatin mRNA**

The regression equation for myostatin content was  $0.992 - 0.24160 \text{ Day} + 0.0266 \text{ Day}^2 - 0.000789 \text{ Day}^3$  ( $P < 0.05$ ,  $r^2 = 0.413$ ). Figure 1-A depicts the patterns of steady-state levels for myostatin mRNA during chicken embryonic development. Myostatin gene expression was first seen during the blastoderm stage of the chick embryo (unincubated embryo, E 0) and remained constant through E 1. Myostatin mRNA dramatically declined on E 2 and remained lower through E 6. Levels then sharply increased on E 7 and plateaued through E 16. Myostatin mRNA increased by E 17 and remained high through E 19, then decreased prior to hatching. The highest and lowest myostatin mRNA levels were seen in the 1- and 2-day-old chicken embryo, respectively.

Although the main source of myostatin is skeletal muscle tissues (McPherron and Lee, 1997), myostatin mRNA expression is certainly not limited to skeletal muscle tissues. Myostatin mRNA was detected in adipose tissue and tubuloalveolar secretory lobules of the lactating mammary gland (Ji et al., 1998). Our finding of strong expression in the early chicken embryo (E 0 and E 1) is intriguing and suggests the possibility that myostatin has an important role during early chicken embryonic development.

The ontogeny of myostatin mRNA (increase in thoracic/abdominal embryo preparations at E 7 coupled with a high level of expression in skeletal muscle at E 17 and the reduction prior to hatching) coincides roughly with the periods of primary and secondary muscle fiber formation. Primary muscle fiber formation occurs by E 7 and secondary muscle fiber formation occurs between E 7 and E 18 in chicken embryos (Feredette and Landmesser, 1991). Because the myofibre number is mostly completed by hatching, the reduction in myostatin mRNA abundance prior to hatching could be due to the reduction in myogenic and mitogenic events. The developmental pattern of chicken myostatin mRNA expression is similar to that reported for cattle (Kambadur et al., 1997) and for pigs (Ji et al., 1998).

### **Follistatin mRNA**

The regression equation for follistatin was  $0.07026 + 0.05322 \text{ Day}$  ( $P < 0.0001$ ,  $r^2 = 0.801$ ). In figure 2-A, the steady-state levels of follistatin mRNA during chicken embryonic development are shown. Follistatin gene expression was first seen during the blastoderm stage of the chick embryo (unincubated embryo, stage 1, E 0). Overall follistatin mRNA increased about 6 fold from E 1 to E 20 of embryonic development. Follistatin mRNA levels decreased from E 0 to E 1 and remained low through E 5, then

increased on E 6 and plateaued through E 9. Follistatin mRNA increased on E 10 (~ 2 fold) and were followed by an additional increase on E 13 and E 14. The same pattern as seen between E 10 and E 14 was seen again between E 15 and E 19. Follistatin mRNA then reached the highest levels prior to hatching. The lowest mRNA expression was seen in the 3-day-old chick embryo.

Follistatin is ubiquitously expressed in rats (Tuuri et al., 1994) and chicken testes and ovulatory follicles (Davis and Johnson, 1998). Early expression of the follistatin gene (E 0) could be due to early embryonic developmental events. For instance, BMPs have ventralizing properties, which result in the ectoderm displaying epidermal characteristics. However, follistatin was able to prevent ventralization by the BMPs and allow the ectoderm to follow a neural fate (Hemmati-Brivanlou et al., 1991).

Mice over-expressing follistatin had reduced fertility (Matzuk et al., 1996). Males had smaller testes and females had smaller ovaries and thinner uteri, which suggests that follistatin has a key role during sexual development. This could explain our findings of an increase in follistatin mRNA levels between E 6 and E 9, which corresponds to the time of sexual differentiation in chick embryos.

### **Activin-B mRNA**

The regression equation for activin-B is  $0.2904 + 0.0659 \text{ Day} - 0.00347 \text{ Day}^2$  ( $P < 0.05$ ,  $r^2 = 0.359$ ). In figure 2-B, the expression pattern of activin-B during chicken embryonic development is shown. Activin-B gene expression was first seen during the blastoderm stage of the chick embryo (unincubated embryo, stage 1, E 0). Although activin-B mRNA from the whole embryo preparations fluctuated, it varied as the embryo matured. The lowest mRNA level was seen on E 5, after which levels gradually increased

and reached the highest level at E 11. Overall activin-B gene expression from E 11 to E 20 appeared to decline (~ 3.5 fold).

Activins inhibit *in vitro* differentiation of myoblasts, while follistatin acts as a local modulator to prevent this myogenic repression (Link and Nishi, 1997). Our results were in partial agreement with these *in vitro* findings. For example, follistatin mRNA increased from E 10 to E 20, while overall activin-B mRNA appeared to decrease accordingly during this time period. On the other hand, follistatin mRNA increased from E 10 to E 13 when the hypertrophic muscle growth is dominant. Our observation that activin-B mRNA levels were not concomitantly low between these days suggests that the actions of follistatin were not simply reciprocal to those of activins.

### **$\beta$ -Actin mRNA**

The regression equation for  $\beta$ - Actin was  $279.067 + 32.256 \text{ Day} - 2.649 \text{ Day}^2 + 0.06716 \text{ Day}^3$  ( $P < 0.0001$ ,  $r^2 = 0.702$ ). Figure 1-B demonstrates the expression patterns of  $\beta$ -actin mRNA during chicken embryonic development.  $\beta$ -Actin was used as a house-keeping gene and appeared to be stable throughout chicken embryonic development.

Since we made no attempt to determine protein levels in the tissues used in this study, nothing can be concluded on the translation of the myostatin, follistatin and activin-B transcripts detected. However, based on the gene expression results, myostatin could be a significant player in prenatal chicken skeletal muscle growth as well as growth of whole embryos prior to when myogenic identity was established. Therefore, complete understanding of the biochemistry and physiology of myostatin during early embryonic development could be beneficial to human health and food animal agriculture. Follistatin and activin-B were found to be clearly among the determinants of prenatal chicken

muscle growth. Previous experiments demonstrated that the effects of activins and TGF- $\beta$ s on muscle development were distinct. Therefore, expression of TGF- $\beta$ s in chicken embryonic development could be of interest as a key regulator of these processes.

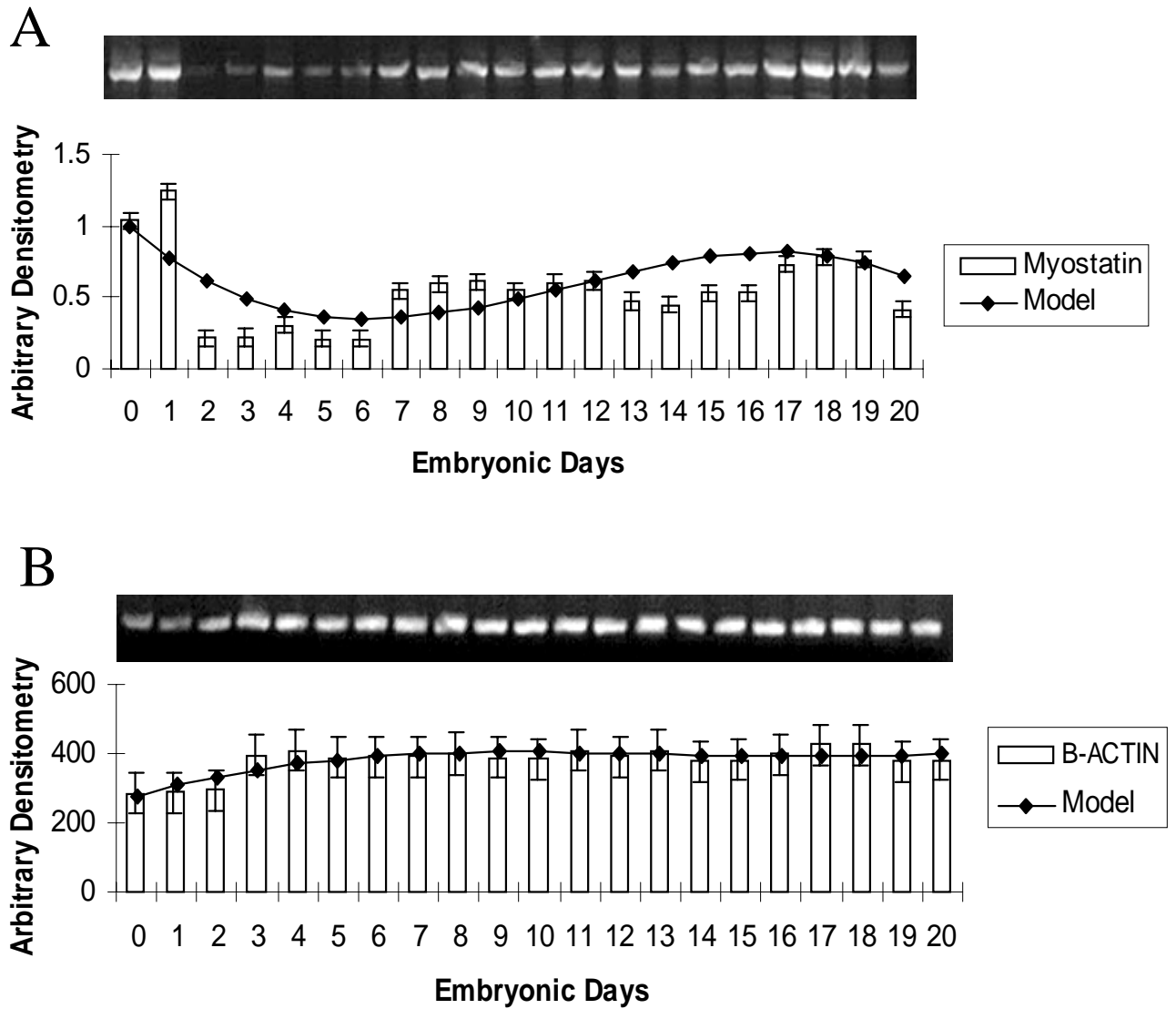


Figure 1. Steady-state levels of myostatin (A) and  $\beta$ -actin (B) mRNA in whole embryo and pectoralis muscle during chick embryo development (n= 6 or 4 per day, respectively). The bands for myostatin on the photograph were scanned by densitometer and the integration values (mean  $\pm$  SD), after normalization to  $\beta$ -actin, are expressed in arbitrary densitometric units at each day.

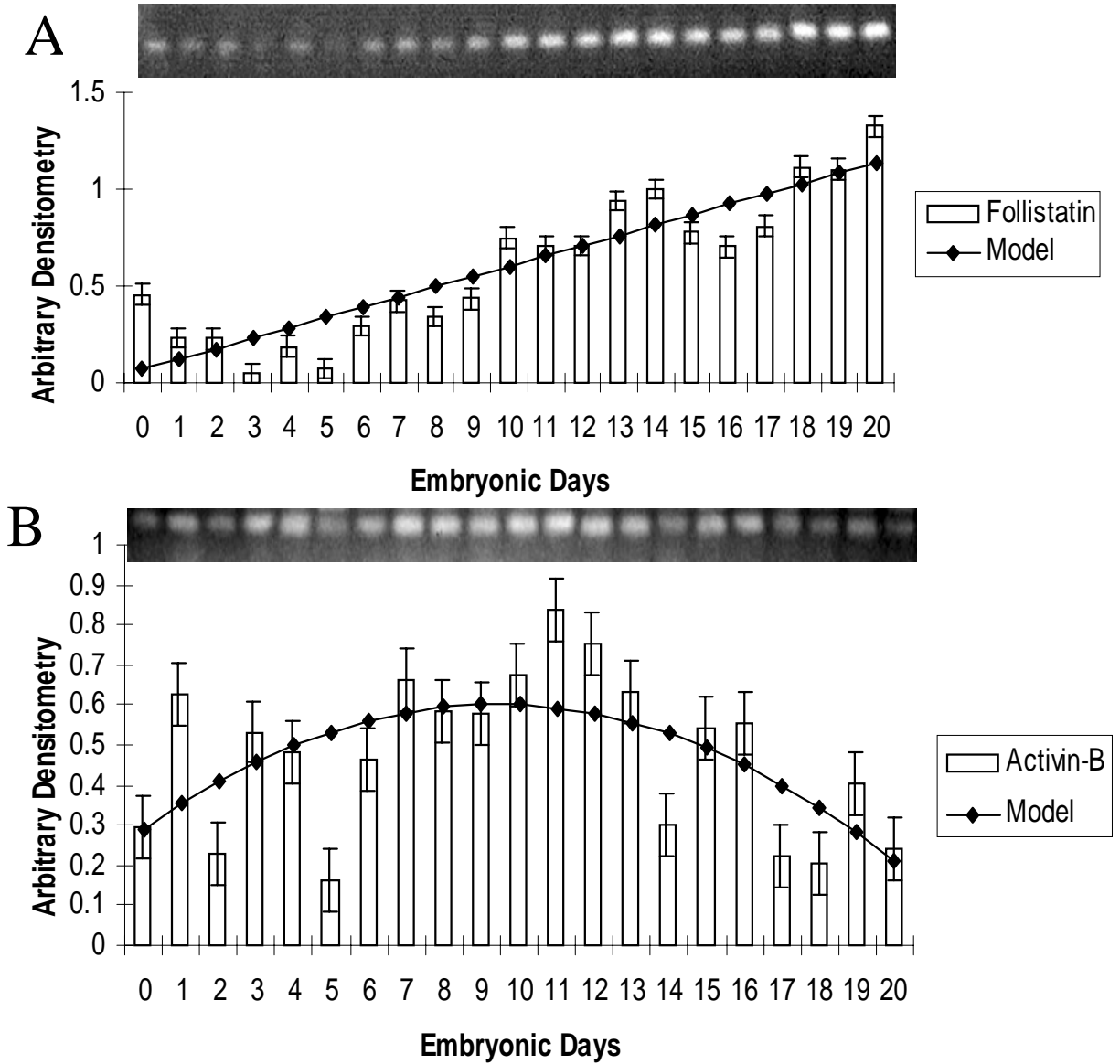


Figure 2. Steady-state levels of follistatin (A) and activin-B (B) mRNA in whole embryo and pectoralis muscle during chick embryo development (n= 6 or 4 per day, respectively). The bands for follistatin and activin-B on the photograph were scanned by densitometer and the integration values (mean  $\pm$  SD), after normalization to  $\beta$ -actin, are expressed in arbitrary densitometric units at each day.

## Chapter 2

### **THE TEMPORAL EXPRESSION OF GROWTH FACTOR GENES DURING MYOGENESIS OF SATELLITE CELLS DERIVED FROM THE BICEPS FEMORIS AND PECTORALIS MAJOR MUSCLES OF THE CHICKEN**

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## INTRODUCTION

Satellite cells, which lie between the basal lamina and sarcolemma of the muscle fiber (Mauro, 1961) are the myogenic stem cells of vertebrate skeletal muscle. They are responsible for the processes of growth and repair in postnatal muscle. These cells are considered mitotically quiescent until stimulated by growth factors to proliferate, differentiate and fuse to existing muscle fibers (for review, see Dodson et al., 1996). Among the most studied growth factors that have specific actions on the proliferation and differentiation of myoblasts are transforming growth factor-betas (TGF- $\beta$ ), insulin-like growth factors (IGF), and fibroblast growth factors (FGF). The IGFs and FGFs are actively involved in control of proliferation and differentiation of several myogenic cell lines. For example, IGFs stimulated the proliferation of chicken (Duclos et al., 1991) and fish (Venkateswaran et al., 1995) satellite cells and also stimulated the proliferation and differentiation of bovine-derived satellite cells (Greene and Allen, 1991). The mitogenic effects of IGF-I and FGF on turkey embryonic myoblasts and satellite cells were synergistic (McFarland et al., 1993), whereas the effects of these mitogens on chicken satellite cells were only additive (Wilkie et al., 1995).

Myostatin, a recently identified member of TGF- $\beta$  family, has been shown to negatively regulate skeletal muscle growth (McPherron et al., 1997). Myostatin null mutation mice showed a dramatic increase in skeletal muscle mass, primarily due to increased number of muscle fibers (McPherron et al., 1997). Additionally, Carlson et al. (1999) demonstrated that there was a positive correlation between abundance of myostatin mRNA and several different muscles that predominantly express white myosin

heavy chain proteins, particularly type IIb. Activins, which are also members of the TGF- $\beta$  family, have multiple biological effects in a wide variety of reproductive and non-reproductive tissues (for review see, Ying et al., 1997). Activins recently have been shown to inhibit *in vitro* myoblast differentiation, while follistatin, an antagonist of activins (for reviews see, Michel et al., 1993, Patel, 1998), acts as a local modulator to prevent myogenic repression (Link and Nishi, 1997). Additionally, we have shown the developmental pattern of myostatin, activin-B and follistatin gene expression during chicken embryonic skeletal muscle development (Kocamis et al., 1999). Since satellite cells are the major component of postnatal skeletal muscle growth, we wanted to determine if satellite cells derived from two different muscle fiber-type sources express these recently identified TGF- $\beta$  family members myostatin, activin-B, and follistatin, and whether the expression of these genes was regulated during myogenesis.

Satellite cells derived from the chicken *pectoralis major* (predominantly white fibers, PM) and *biceps femoris* (predominantly red fibers, BF) muscles showed differences in metabolic variables, and mitogenic responses to various concentrations of chicken serum (McFarland et al., 1997). For instance, BF satellite cells were more responsive to the mitogenic effects of chicken serum than PM satellite cells in the chicken; PM satellite cells, however, differentiated faster than BF satellite cells when induced to differentiate by administration of low-serum containing medium (McFarland et al., 1997). For this reason, the objective of the present study was to evaluate whether differences in gene expression for growth factors (TGF- $\beta$ 2, IGF-I, -II and basic, bFGF) could explain the variation in properties of satellite cells isolated from different chicken muscle types.

## MATERIALS AND METHODS

### Cell isolation and culture

Satellite cells were isolated from the *pectoralis major* and *biceps femoris* muscles of 5-week-old female Cornish Rock broiler chickens as previously described (McFarland et al., 1997). Preliminary studies (data not shown) indicated that approximately 90% of primary broiler chicken satellite cells stained positive for the muscle-specific marker, desmin. Following proliferation for 6-7 days, these cultures were reduced to less than 30% desmine positive cells. Because of greatly diminished myogenicity of these primary cultures, satellite cells from both the *pectoralis major* and *biceps femoris* were cloned to produce pure myogenic cultures. Following cloning, only one clone from each muscle source exhibited  $\geq 50\%$  fused cells when administered low serum-containing medium for 2 days. The remainder of clones fused to form cultures with a lowered percentage of nuclei within myotubes. We, therefore, chose the *biceps femoris* clone BBF9 and *pectoralis major* clone BPM8 for these studies. Cells were plated in 24-well plates at a density of 7600 cells/well in DMEM + 10% chicken serum (CS) + 5% horse serum (HS) and allowed to attach for 15 h in a CO<sub>2</sub> incubator at 38.5C. Cells were then administered McCoy's 5A + 10% CS + 5% HS + 10 ng/ml basic fibroblast growth factor (bFGF) daily for 3 days. Following this, cells were administered DMEM + 3% HS + 1 mg/mL gelatin (fusion medium) daily for 3 days. Measurements of DNA (5 well replicates), creatine kinase (5 well replicates) and RNA (3 replicates with 6 wells pooled/replicate) were made at each time point.

DNA was quantified in wells by the use of a fluorescence plate reader as previously described (McFarland et al., 1995). Differentiation was measured by

determination of muscle specific creatine kinase levels using a plate reader. The procedure was adapted from the method of Shainberg et al. (1971) as modified by Florini (1989) using thio-NAD. Cell samples for RNA isolation were rinsed with PBS, trypsinized from the substratum, quenched with soybean trypsin inhibitor and the cells from each row (6 wells) were pooled for each of the 3 replicate samples. The suspensions were centrifuged in microfuge tubes at 800 x g for 15 min at 4C, the supernatants discarded, and the pellets frozen at -90C until assay.

### **RNA extraction**

Total RNA was extracted from harvested cells at all of the time points mentioned above using the Tri-Reagent (Sigma, St. Louis, MO) modification of the guanidine isothiocyanate/phenol-chloroform method as described by Chomczynski and Sacchi (1987). The RNA concentration was estimated by absorbance at 260 nm in a Shimadzu spectrophotometer (Model UV-1201, Columbia, MD). The integrity of the RNA was assessed by UV visualization of ethidium-bromide stained intact 28S and 18S bands on a mini-agarose gel. Samples of RNA were stored at -80 C.

### **RT-PCR**

Reverse transcription (RT) was performed by adding 2 µg of total RNA to 2 µg of oligo dT primers and sterilized nuclease-free dd H<sub>2</sub>O in a final volume of 15 µl. The samples were heated at 70 C for 5 minutes and then immediately cooled to 4 C for 2 minutes. Reverse transcription buffer containing dNTPs (final concentration of each was 10 mM), 25 units of RNase inhibitor, and 200 units of murine maloney leukemia virus reverse transcriptase (Promega, Madison, WI) were added to each sample. The sample, with a final volume of 40 µl, was incubated at 37 C for 1 hr followed by a 5 min

incubation at 95 C. For the PCR reaction, 2  $\mu$ l of RT reaction mixture were added to 48  $\mu$ l of solution containing 5  $\mu$ l of Taq buffer, 1  $\mu$ l Taq DNA polymerase (Display Systems Biotech, Vista, CA), 1  $\mu$ l dNTPs (final concentration of each was 10 mM), 1  $\mu$ l each of forward and reverse primers and 41  $\mu$ l sterile nuclease-free dd H<sub>2</sub>O. The PCR reaction started with one cycle consisting of 94 C for 5 min, an annealing step of 65 C (for bFGF and activin-B) or 55 C (for IGF-I, myostatin, follistatin and  $\beta$ -actin) for 1 min and extension at 72 C for 1 min. The first cycle was followed by 30 cycles (25 cycles for  $\beta$ -actin) consisting of 30-sec intervals of 94 C, followed by 65 C for bFGF and activin-B, or 55 C for IGF-I, myostatin, follistatin and  $\beta$ -actin, followed by 72 C. To establish a linear range of amplification for each gene, several different cycle numbers of PCR (10, 15, 20, 25, 30 and 35 cycles) were run. For IGF-II and TGF- $\beta$ 2, touchdown PCR was run. This program consisted of a 5 min, 94C denaturation step, followed by 5 cycles in which the initial annealing temperature of 72C for IGF-II and 65 for TGF- $\beta$ 2 was reduced by 1C per cycle, then 30 cycles in which the annealing temperatures were 68C for IGF-II and 61C for TGF- $\beta$ 2. Denaturation, extension and annealing time were programmed as described above. A water (no cDNA) PCR reaction was used as a control for each gene and no contamination of reaction mixture components was found.

### **PCR primers**

All PCR primers were obtained from Gibco BRL Inc. (Grand Island, NY). Primers for myostatin were designed on the basis of published sequences of chicken myostatin (McPherron and Lee, 1997). The sequence of the forward primer was 5' GACTATCATGCCACAACCGAGACGA 3', while the reverse primer was 5' GTGTACCAGGTGAGTGTGCGGGTATT 3'. Forward and reverse primers predicted a

PCR product of 657 base pairs (bp), which corresponded to bases 327-984 of the sequence. Primers for follistatin were designed on the basis of the published sequence of chicken follistatin (Graham and Lumbsden, 1996). The sequence of the forward primer was 5' CATCCCGTGCAAAGAAAC 3', while the reverse primer was 5'CTCGTAGGCTAATCCAATG 3'. These primers amplified a PCR product of 445 bp as previously reported (Davis and Johnson, 1998), which corresponded to bases 260-705 of the sequence. Primers for activin-B were based on a published partial sequence (Mitrani et al., 1990). The sequence of the forward primer was 5' TACTGTGAAGGGAGCTGCCCCG 3', while the reverse primer was 5' GTACAGCATTGACATTGTGC 3'. These primers amplified a PCR product of 162 bp as previously reported (Davis and Johnson, 1998), which corresponded to bases 13-175 of the sequence.

Primers for bFGF and TGF- $\beta$ 2 were designed on the basis of published sequences of chicken bFGF and TGF- $\beta$ 2 (Borja et al., 1993; Burt and Paton, 1991, respectively). Primers for bFGF amplified a PCR product of 270 bp which corresponded to bases 432-701 of the sequence, while primers for TGF- $\beta$ 2 amplified a PCR product of 269 bp which corresponded to bases 6452- 6722 of the sequence. The sequences of forward primers for bFGF and TGF- $\beta$ 2 were 5' GATCCGCACATCAAACACTGC 3', 5' AGGAATGTGCAGGATAATT 3', while the reverse primers were 5' GATACGTTTCTGTCCAGGTCC 3', 5' ATTTTGGGTGTTTTGCCAA 3', respectively. Primers for IGF-I and IGF-II were designed on the basis of published sequences of chicken IGF-I and IGF-II (Kajimoto and Rotwein, 1989; Darling and Brickell, 1996, respectively). The sequences of forward primers for IGF-I and IGF-II were 5'

GTATGTGGAGACAGAGGCTTC 3', 5' TGTGGAGGAGTGCTGCTTTC 3', while the reverse primers were 5' TTTGGCATATCAGTGTGGCGC 3', 5'

GGGAGGTGGCGGAGAGGTCA 3', respectively. Forward and reverse primers amplified a PCR product of 200 bp for IGF-I which corresponded to bases of 439-638, while primers for IGF-II amplified a 101 bp PCR product which corresponded to bases of 44-145. Primers for  $\beta$ -actin were used to amplify a 285 bp product as previously published (Yamamura et al., 1991), as an internal standard for normalizing the level of amplification. The sequence of the forward primer was 5' TCATGAAGTGTGACGTTGACATCCGT 3', while the reverse primer was 5' CCTAGAAGCATTGCGGTGCACGATG 3'.

The amplified PCR products for each gene were visualized on 1.5 % agarose gels stained with ethidium bromide. Products were analyzed by densitometry of stained gels, and data should, therefore, be considered on a semi-quantitative basis. The identity of all PCR products was confirmed by sequence analysis.

### **Statistical analysis**

Analysis of variance was performed by the GLM procedure of SAS<sup>®</sup> (SAS Institute, 1989). Duncan's New Multiple Range test was used to compare mean values of PM and BF satellite cell DNA and creatine kinase ratios. Statements of significance were based on  $P < 0.05$  unless otherwise noted.

## RESULTS

Proliferation rates and differentiation kinetics of PM and BF satellite cells are shown in Figure 1 (A and B, respectively). Proliferation rates of BF satellite cell clone used were greater than PM clone. However, PM satellite cells began differentiation sooner than BF satellite cells.

### **$\beta$ -Actin mRNA**

Figure 2 depicts the patterns of  $\beta$ -Actin gene expression in both cell cultures.  $\beta$ -Actin was used as a housekeeping gene and was stable throughout the experiment in both cultures.

### **TGF- $\beta$ Family mRNA**

Representative patterns of steady-state levels for myostatin and TGF- $\beta$ 2 mRNA during PM and BF chicken satellite cell myogenesis are shown in figure 3. No myostatin gene expression was detected in either satellite cell culture at 0 h and 24 h after plating. The ontogeny of myostatin gene expression was nearly identical in both cell cultures. However, myostatin mRNA level increased at 72 h after plating when fusion starts in BF satellite cells (Fig. 3,  $P < 0.01$ ), whereas there was no significant change in PM satellite cells at this time. Myostatin mRNA, once it reached the highest value at 72 h, plateaued through 144 h in both cell cultures (Fig. 3). TGF- $\beta$ 2 mRNA was first detected in 0 h satellite cells derived from either PM or BF muscle and remained constant until 48 h culture. Amounts then sharply increased (Fig. 3,  $P < 0.01$ ) and remained constant until the end of culture period in both PM and BF satellite cells. The patterns of activin-B and follistatin gene expression in both cell cultures are shown in Figure 4. Activin-B and



follistatin mRNAs were undetectable at 0 h in both satellite cell cultures. Activin-B mRNA level in PM satellite cells was higher than in BP satellite cells at 72 h and 120 h (Fig. 4,  $P < 0.01$ ), whereas levels in BF satellite cells were higher than in PM satellite cells at 96 h and 144 h (Fig. 4,  $P < 0.01$ ). Amounts of follistatin mRNA in PM satellite cells were higher than in BF satellite cells at 24, 96, and 120 h culture (Fig. 4,  $P < 0.01$ ). However, amounts in BF satellite cells were higher than in PM satellite cells at 144 h after plating ( $P < 0.01$ ).

### **IGF-I, -II and bFGF mRNA**

Figure 5 shows the patterns of IGF-II and bFGF gene expression in both cell cultures. Although, using the same primer sequences, Burt et al. (1992) demonstrated IGF-I mRNA expression in different chicken tissues, no IGF-I gene expression was detected in either cell culture at any time point in present study (data not shown). IGF-II mRNA level plateaued in PM satellite cells by 48 h after plating ( $P < 0.05$ ), and remained elevated until 144 h of culture period. However, levels in BF satellite cells dramatically declined at 96 h after plating ( $P < 0.01$ ) and remained reduced until 144 h. No bFGF mRNA was detected at 0 h satellite cells derived from either PM or BF muscle (Fig. 5). bFGF gene expression in both satellite cell cultures increased at the 72 h culture period ( $P < 0.05$ ) and remained at this level in BF satellite cells through 144 h. Although bFGF mRNA in PM satellite cells increased at 144 h with respect to 120 h of culture ( $P < 0.01$ ), amounts did not differ between PM and BF satellite cells at this time period (Fig. 5).

## DISCUSSION

The current study was conducted to evaluate differences in properties of satellite cells isolated from different chicken muscle types. Clonal-derived satellite cell cultures were used to eliminate the interference associated with nonmyogenic cell contamination such as fibroblasts. It is possible that other clones within a given muscle may exhibit variation in the gene expression of the various proteins we analyzed. However, studies to examine this possibility will require improvements to increase the efficiency of cloning of broiler chicken satellite cells. We determined that BF clone was more responsive to mitogenic stimuli of serum than PM clone. However, PM satellite cells more rapidly fused to form multinucleated myofibers than BF satellite cells. These findings were in agreement with the results obtained by McFarland et al. (1997). On the other hand, the observations seen with turkey BF and PM satellite cells, in which PM satellite cells were more responsive to the mitogenic effects of serum (McFarland et al., 1995), were in contrast to the results obtained from the present and previous (McFarland et al., 1997) experiments. The reasons for these differences between species remain unclear.

TGF- $\beta$ s inhibit proliferation and differentiation of satellite cells (Allen and Boxhorn, 1987, 1989) as well as embryonic myoblasts in culture (Massague et al., 1986). Lafyatis et al. (1991) demonstrated that TGF- $\beta$ 2 mRNA level increased in C2C12 myoblasts (cell line derived from satellite cells) when cells were allowed to differentiate in low serum medium. In the present study, TGF- $\beta$ 2 mRNA level in both cultures, however, reached the highest level before fusion started (72 h after plating) and remained constant throughout the experiment. Also, the ontogeny of TGF- $\beta$ 2 gene expression in

both cultures did not differ. Therefore, we suggest that TGF- $\beta$ 2 may not be involved in the various response of PM and BF satellite cells to different serum concentrations.

Carlson et al. (1999) suggested that the higher concentrations of myostatin mRNA in white muscles may function as an inhibitor of satellite cell proliferation. This speculation is consistent with several previous experiments. First, myostatin knockout mice showed muscle hyperplasia and hypertrophy (McPherron et al., 1997), both of which do not occur if satellite cell proliferation is blocked in normal animals (Gulati, 1987). Second, muscles that predominantly have white fibers have low satellite cell densities compared with muscles that predominantly have red fibers, which coincides with the higher amount of myostatin expression in white muscles (Carlson et al., 1999; Wehling et al., 2000). Third, animals subjected to hind limb unloading which causes suppression of satellite cell proliferation (Darr and Schultz, 1989) demonstrated high level of myostatin expression (Carlson et al., 1999). However, our results did not support the speculation suggested by Carlson et al. (1999), because myostatin gene expression was nearly identical in both PM and BF satellite cell cultures. The conclusions drawn by Carlson et al. (1999) were based on whole muscle analyses, which included not only satellite cells but also a major contribution of mRNA originating from myonuclei. Myostatin levels within the muscle proper may differ from the levels found in pure satellite cell population. On the other hand, myostatin proteins with different molecular masses were found in the plantaris (predominantly red fibers) and soleus (predominantly white fibers) muscles of rats (Wehling et al., 2000) suggesting that alternative posttranslational modifications of myostatin occur in different muscle fibers. Since myostatin gene expression in PM and BF satellite cells used in the present study was not

different, it would be of interest to know whether the same myostatin protein modifications take place in satellite cells derived from PM and BF muscles and whether they are similar to those found in mature muscle.

Activins are covalently-linked dimers of two distinct  $\beta$  subunits, thus existing in three different forms, activin-A, AB and B (Ying, 1987), whereas follistatin is a monomeric glycosylated protein present in several isoforms (for reviews see, Michel et al., 1993; Patel, 1998). Most, if not all, of the biological actions of activins are neutralized by its binding to follistatin (reviewed in Michel et al., 1993). Follistatin is also believed to interact with, and possibly regulate activities of, members of the TGF- $\beta$  superfamily. Activin and follistatin mRNAs (Kocamis et al., 1999) and protein (Link and Nishi, 1997) expression have been shown in vivo during chicken pectoral muscle development and in cultures derived from this muscle group. Activins inhibited pectoral muscle cell differentiation in culture, while follistatin stimulated this processes (Link and Nishi, 1997). Also, it was shown that the effects of activin on muscle cell differentiation were different than those of TGF- $\beta$ s in vitro (Link and Nishi, 1997). Consistent with the latter findings, the pattern of activin-B gene expression from both satellite cultures was different from that of TGF- $\beta$ 2 in the current study. Furthermore, overall activin-B and follistatin mRNA expression patterns (i.e. both increased at 72 h after plating when fusion started) found in PM satellite cells may contribute to the inhibitory functions of follistatin on activins. For these same genes, the parallel expression patterns, however, did not exist in BF satellite cells in culture. Therefore, it is suggested that follistatin may predominantly interact with either other activin isoforms or other TGF- $\beta$  superfamily members, such as bone morphogenic proteins.

Basic fibroblast growth factor (bFGF) was chosen to represent the FGF family gene expression, because it was shown to be a more potent mitogen than acidic FGF for chicken satellite cells (Wilkie et al., 1995). Moore et al. (1991) observed that FGF mRNAs and their receptor were down-regulated during myoblast differentiation in culture, and suggested that FGFs acted in an autocrine fashion to control myogenesis. This hypothesis was supported by a study that demonstrated that myoblasts transfected with an FGF antisense expression vector differentiated faster than control transfected cells (Fox et al., 1994). On the contrary, amount of bFGF mRNA in the present study were increased during differentiation and remained high in both satellite cell cultures. Although FGF receptor numbers and affinities were similar in turkey PM and BF satellite cells (McFarland et al., 1997), this may not be the case in chicken due to the different response of these satellite cells to various concentrations of serum.

It has been shown that exogenous IGFs (IGF-I and -II) stimulated the proliferation of chicken (Duclos et al., 1991) and turkey (McFarland et al., 1993) satellite cells in vitro, whereas they did not stimulate the differentiation of turkey satellite cells (McFarland et al., 1993). Based on the report by Florini et al. (1991) that high-IGF-producing rodent myoblast cell lines appeared relatively insensitive to exogenous IGFs, it was speculated that the high amount of endogenous IGFs produced by turkey satellite cells may be sufficient to allow maximal cell differentiation. However, this speculation was disproved by Ernst et al. (1996) who showed that turkey satellite cells did not express the IGF-I gene and that IGF-II production by these cells was highest in proliferating cells and significantly decreased during differentiation. Our results that IGF-I gene expression was not detected in either chicken PM or BF satellite cell cultures

were consistent with results obtained by Ernst et al. (1996). On the other hand, IGF-II gene expression in the current study was highest at 72 h after plating when fusion started and remained high throughout the culture period in PM satellite cells. Given the fact that IGF-I and IGF-II only act through the type-I receptor on chicken (Duclos et al., 1991) and turkey (Sun et al., 1992) satellite cells, IGF-II may have autocrine/paracrine functions in muscle development, whereas IGF-I may predominantly act in an endocrine fashion.

Because of the lack of specific antibodies to the chicken gene products, no attempt was made to determine amount of protein in cell cultures used in the present study. Therefore no conclusions can be drawn as to the translational efficiency or modifications of the growth factor transcripts studied. Additionally, the genes examined in the present study were not conducive for the design of a multiplex PCR assay which would have yielded quantitative data. Our results should, thus, be considered on a semi-quantitative basis in light of the observed expression patterns for the various growth factor genes. It is likely that changes in the proteins for the individual genes will roughly parallel their mRNA patterns. As indicated earlier, posttranslational modifications to the proteins may have a significant effect on their activities and ultimately on the cellular response.

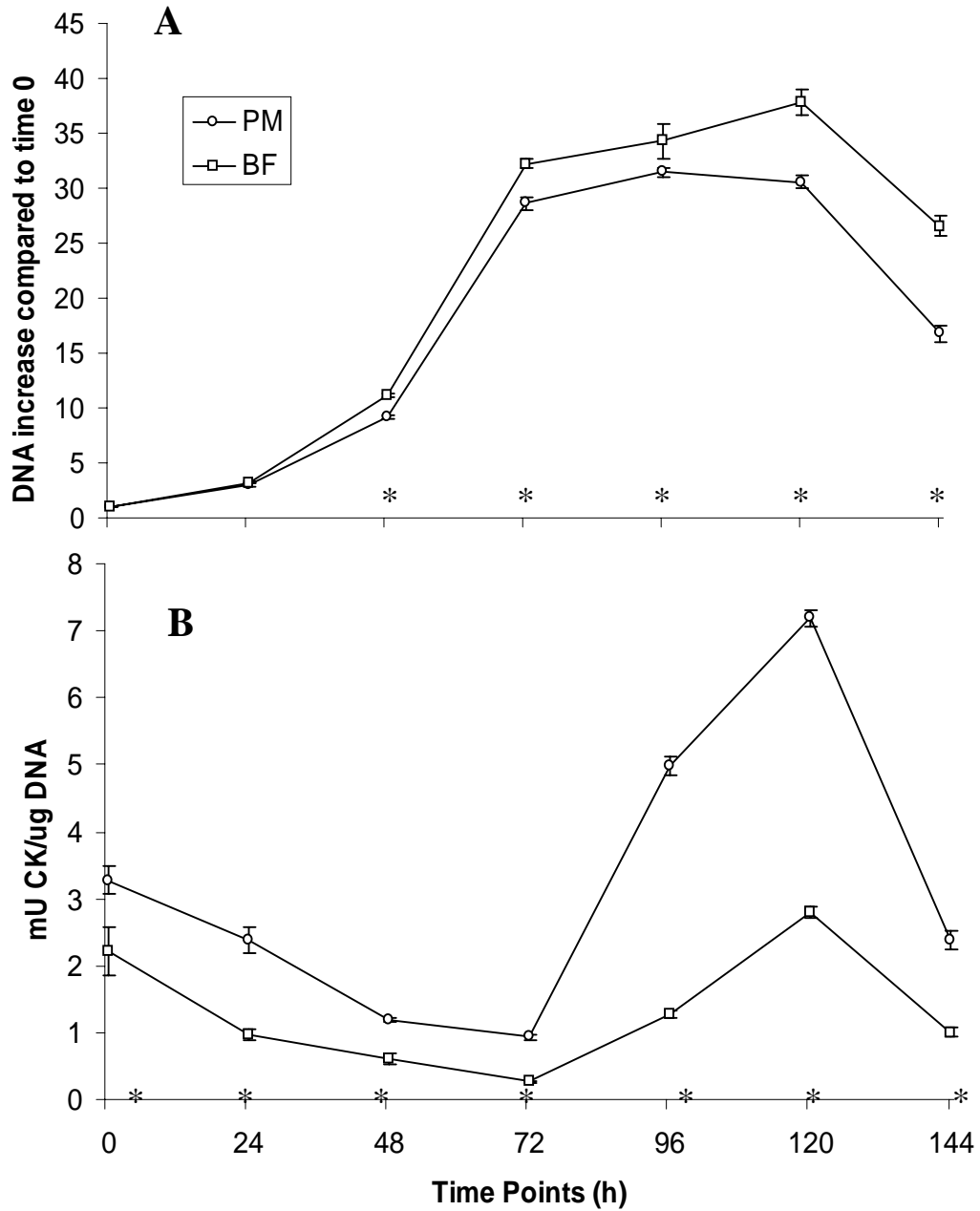


Figure 1. Comparison of the proliferation rates (A) and differentiation kinetics (B) of PM and BF satellite cells. Cultures were evaluated as described in materials and methods. Asterisks indicate when proliferation and differentiation levels were significantly different ( $P < 0.05$ ).

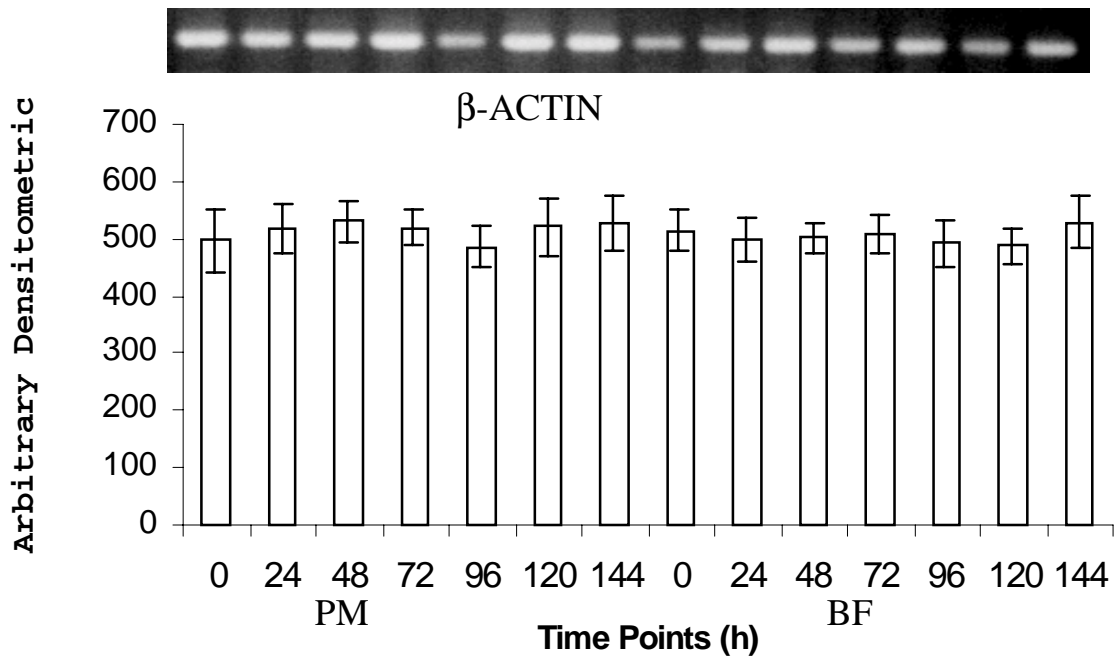


Figure 2. Representative steady-state levels of  $\beta$ -actin mRNA in in vitro PM and BF satellite cell myogenesis (n= 3, per time point). The bands for  $\beta$ -actin mRNA were analyzed by densitometry and the integration values (mean  $\pm$  SD) were expressed in arbitrary densitometric units at each time point. Means with the same superscripts were not significantly different.



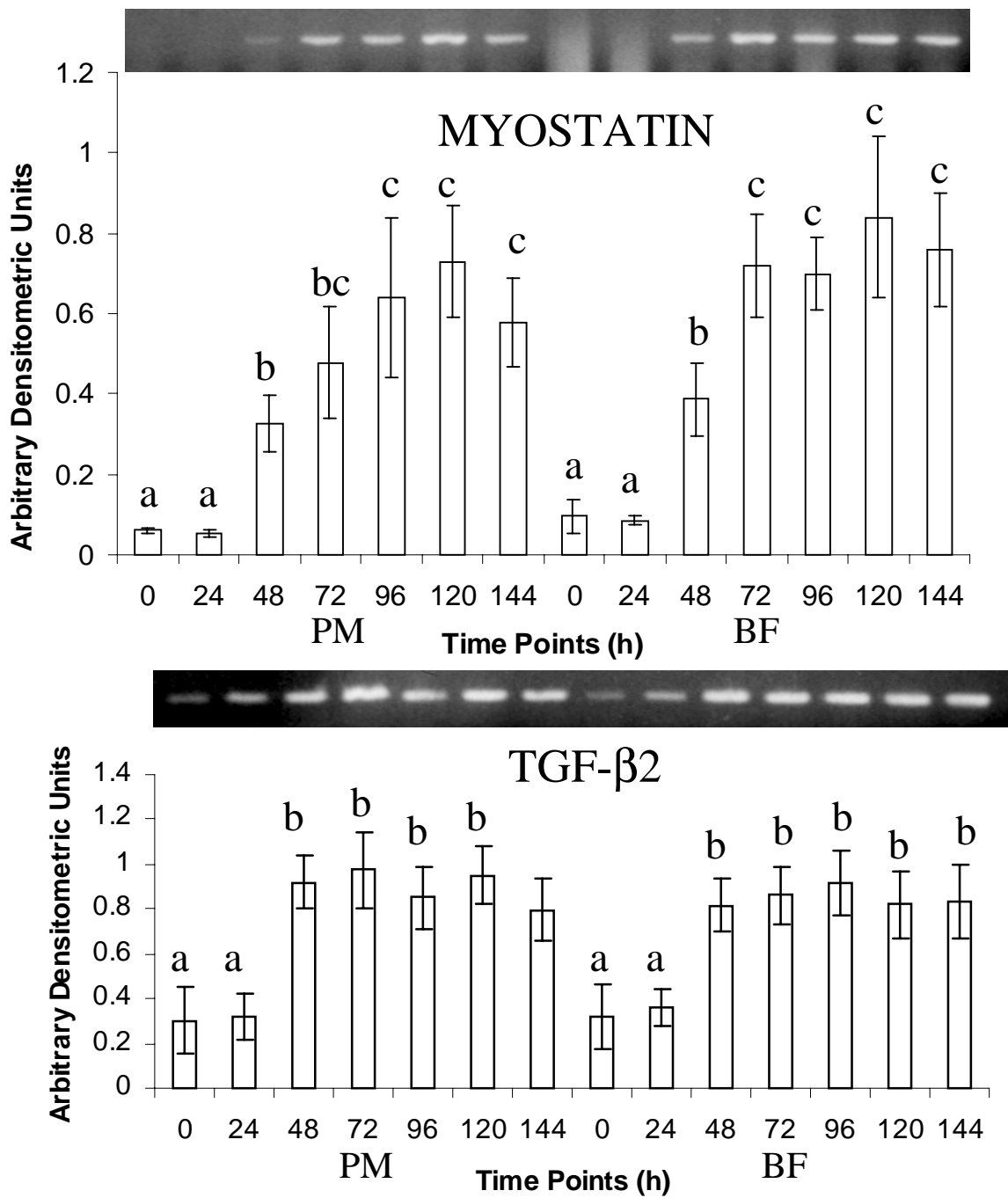


Figure 3. Representative steady-state levels of myostatin and TGF- $\beta$ 2 mRNA in vitro PM and BF satellite cell myogenesis (n= 3, per time point). The bands for myostatin and TGF- $\beta$ 2 were analyzed by densitometry and the integration values (mean  $\pm$  SD), after normalization to  $\beta$ -actin, are expressed in arbitrary densitometric units at each time point. Means with the same superscripts were not significantly different.

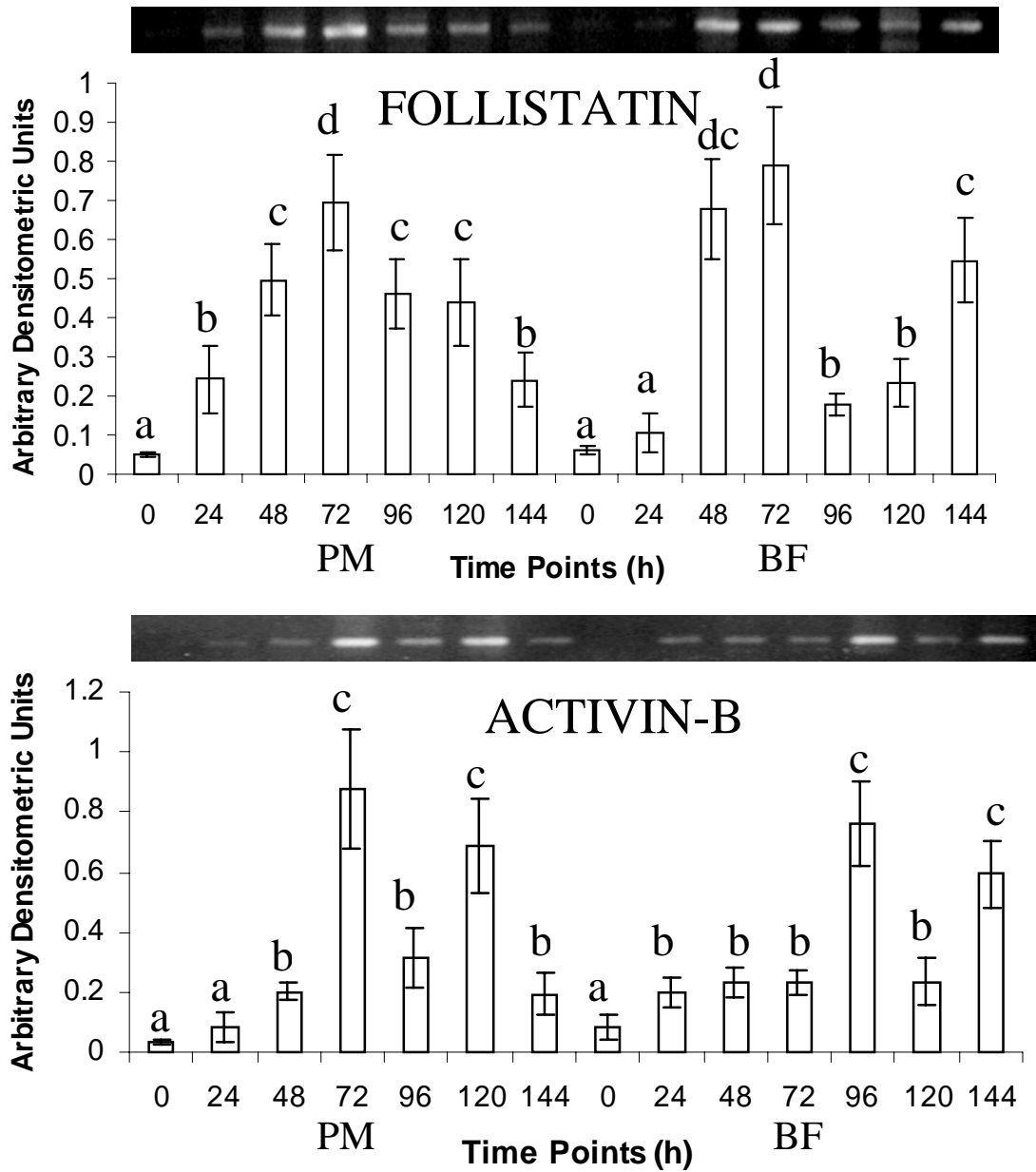


Figure 4. Representative steady-state levels of follistatin and activin-B mRNA in in vitro PM and BF satellite cell myogenesis (n= 3, per time point). The bands for follistatin and activin-B were analyzed by densitometry and the integration values (mean ± SD), after normalization to  $\beta$ -actin, are expressed in arbitrary densitometric units at each time point. Means with the same superscripts were not significantly different.

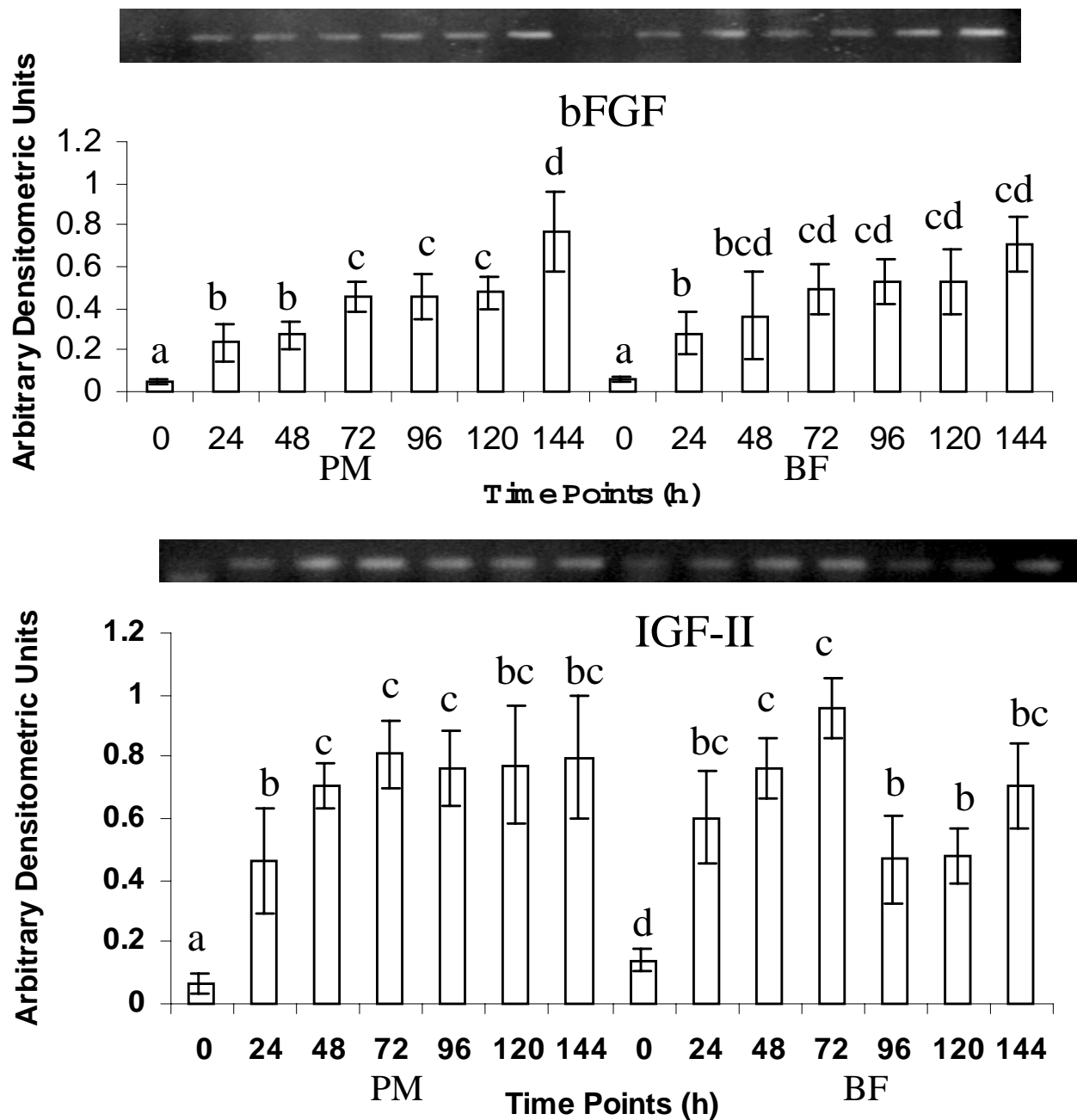


Figure 5. Representative steady-state levels of IGF-II and bFGF mRNA in in vitro PM and BF satellite cell myogenesis (n= 3, per time point). The bands for IGF-II and bFGF were analyzed by densitometry and the integration values (mean ± SD), after normalization to  $\beta$ -actin, were expressed in arbitrary densitometric units at each time point. Means with the same superscripts were not significantly different.

## Chapter 3

### **MYOSTATIN, FOLLISTATIN, ACTIVIN-B AND TGF- $\beta$ 2 GENE EXPRESSION PATTERNS IN RESPONSE TO *IN OVO* ADMINISTRATION OF rhIGF-I DURING CHICKEN EMBRYONIC DEVELOPMENT**

## INTRODUCTION

Insulin-like growth factors (IGF) and transforming growth factor-betas (TGF- $\beta$ ) are the most studied growth factors that have specific actions on the proliferation and differentiation of myoblasts. Exogenous IGFs stimulated both proliferation and differentiation of several myogenic cell lines (Florini et al., 1991), while TGF- $\beta$ s inhibited myogenesis in those cells albeit with various magnitude (Florini et al., 1986, Massague et al., 1986). Additionally, *in vitro* studies indicated that IGF-I negatively controlled TGF- $\beta$  expression during early myogenesis (Bosche et al., 1995).

Myostatin, a recently identified member of the TGF- $\beta$  family, has been proposed as a negative regulator of skeletal muscle growth. The ontogeny of myostatin gene expression during chicken embryonic development suggested that myostatin could be a major determinant in prenatal skeletal muscle growth as well as growth of whole embryos (Kocamis et al., 1999). We have previously demonstrated that *in ovo* administration of recombinant human insulin-like growth factor-I (rhIGF-I, 100 ng/per egg) on embryonic day 3 (E3) significantly increased the postnatal muscle growth of 42-day-old chickens (Kocamis et al., 1998, 2000). Therefore, the objective for this study was to evaluate the impact of *in ovo* administration of rhIGF-I on myostatin, activin-B (member of the TGF- $\beta$  family), follistatin (an antagonist of activins), and TGF- $\beta$ 2 gene expression during chicken embryonic development with emphasis on skeletal muscle development.

## MATERIALS AND METHODS

### Injection Procedure

Fertilized eggs (Ross x Ross) were obtained from Wampler-Longacre (Moorefield, WV). The injection procedure was described previously (Kocamis et al., 1998). Briefly, 100 ng/100  $\mu$ L per egg of rh IGF-I (R & D Systems, Minneapolis, MN) in 10 mM acetic acid, 0.1 % BSA was administered with a 22-gauge needle through the blunt end of the egg on embryonic day 3. Prior to injection, the blunt end of the egg was sterilized with 70% ethanol. A dental drill bit was used to create a single hole, without penetrating the chorio-allantoic membrane. The hole was sealed with an adhesive sticker. Because no difference was previously found between vehicle (10 mM acetic acid, 0.1 % BSA) injected and uninjected groups (Kocamis et al., 1998, 2000) in terms of postnatal skeletal muscle growth, uninjected eggs were used as control group in the present study. Eggs were set in a Buckeye incubator/hatcher (temperature  $37 \pm .5$  C, humidity 86 to 87%).

### Tissue collection

Embryos and tissues were harvested in compliance with an approved West Virginia University Animal Care and Use Committee Protocol. Tissue collection was performed as previously described (Kocamis et al., 1999). Briefly, all the embryos were isolated and washed free of yolk, albumen and extra-embryonic membranes by sterile nuclease-free water and were staged according to Hamburger and Hamilton (1951). Whole embryos were collected on each embryonic days (E) 0 to 6 (equivalent to stage 1 to stage 29, n= 6 per day). Thoracic/abdominal halves of embryos consisting of the

lumbo-sacral level to the neck without head were collected on each of E 7 and E 8 (stage 31 and stage 34, respectively, n= 6 per day). *Pectoralis* muscle was collected on each of E 9 to E 20 (n= 4 per day). All the tissue collections were performed at consistent times for each sampling day throughout the experimental period, starting day 9, stage 35, and every 24 h until day 20, stage 45.

### **RNA extraction and RT-PCR**

Total RNA was extracted from all of the tissues mentioned above using the Tri-Reagent (Sigma, St. Louis, MO) modification of the guanidine isothiocyanate/phenol-chloroform method (Chomczynski and Sacchi, 1987). The RNA concentration was estimated by absorbance at 260 nm in a Shimadzu spectrophotometer (Columbia, MD). Samples of RNA were stored at -80 C.

Reverse transcription (RT) was performed as described in chapter 1. The PCR reaction started with one cycle consisting of 94 C for 5 minutes, an annealing step of 65 C for myostatin, and activin B, or 55 C for follistatin and  $\beta$ -actin for 1 minute and extension at 72 C for 1 minute. The first cycle was followed by 30 cycles (25 cycles for  $\beta$ -actin) consisting of 30-sec intervals of 94 C, followed by 65 C for myostatin and activin-B or 55 C for follistatin and  $\beta$ -actin, followed by 72 C. For TGF- $\beta$ 2, touchdown PCR was run. This program consisted of a 5-minute, 94 C denaturation step, followed by 5 cycles in which the initial annealing temperature of 65 was reduced by 1C per cycle, then 30 cycles in which the annealing temperature was 61C. Denaturation, extension and annealing time were programmed as described above. To establish a linear range of amplification for each gene, several different cycle numbers of PCR (10, 15, 20, 25 and

30 cycles) were run. As a control, a PCR reaction without c-DNA was run, and no contamination was found in reaction mixture (data not shown).

### **PCR primers**

All PCR primers were synthesized by Gibco BRL Inc. (Grand Island, NY). Primer designs, sequences and expected product sizes for myostatin, activin-B, follistatin and  $\beta$ -actin were described in the chapter 1. Primers for TGF- $\beta$ 2 amplified a PCR product of 269 bp which corresponded to bases 6452- 6722 of the sequence. The sequence of the forward primer for TGF- $\beta$ 2 was 5' GATCCGCACATCAAACACTGC 3', while the reverse primer was 5' ATTTTGGGTGTTTTGCCAA 3'.

The amplified PCR products for each gene were visualized on 1.5 % agarose gels stained with ethidium bromide. Products were analyzed by densitometry of stained gels, and data should, therefore, be considered to be semi-quantitative. The identity of all PCR products was confirmed by sequence analysis. Mean gene expression values for each day were derived from a minimum of four individual sample collections and a minimum of three independent cDNA and RT-PCR amplifications per sample.

### **RESULTS**

Myostatin gene expression was first seen in 4-day-old chicken embryos and then gradually increased through embryonic day 8 (E 8) in both control and *in ovo* rhIGF-I injected groups (Fig. 1). Myostatin mRNA levels from control pectoralis muscle sharply increased at E 9 (~ 3 fold) and remained high through E 12. Values then decreased and remained low until E 16. Myostatin expression subsequently increased (~ 3 fold) and remained high until hatching. Myostatin mRNA from pectoralis muscles of rhIGF-I injected embryos increased on E 10 (~ 2.5 fold) and remained high through E 13,



whereas mRNA from control pectoralis muscles increased at E 9 and remained high until E 12.

Follistatin gene expression was first detected on E 0 (Fig. 2). Follistatin mRNA levels from control embryos gradually increased from E 3 to E 8, whereas levels from rhIGF-I injected group remained relatively constant between these days, with the sudden increase at E 5 (~ 2 fold). Follistatin mRNA levels from pectoralis muscles of rhIGF-I injected group were increased at E 12 (~ 2.5 fold) and reached the highest level at E 13. Levels then gradually declined through E 20. However, follistatin mRNA from control pectoralis muscle remained relatively constant between E 9 and E 15. Levels then sharply decreased at E 16 (~ 3 fold) and followed by sudden increase at E 17 (~ 3.5 fold) and reached the highest level at E 19.

Activin-B gene expression was first seen at E 0 and fluctuated through E 8 in control embryos (Fig. 3). However, levels from rhIGF-I injected group from E 3 to E 8 remained relatively constant. In both control and rhIGF-I injected group, activin-B mRNA levels sharply increased at E 8 (~ 2.5 fold) and remained high through E 13. Levels from control group then decreased (~ 2 fold) and remained relatively constant until E 20 whose expression was the highest in this group. Activin mRNA levels from rhIGF-I injected pectoralis muscle fluctuated from E 14 to E 20, with slight decrease.

TGF- $\beta$ 2 gene expression was first found at E 2 and gradually increased until E 7 in both control and *in ovo* rhIGF-I injected chicken embryos (Fig. 4). TGF- $\beta$ 2 mRNA levels from control pectoralis muscle remained relatively constant from E 9 to E 12, then dramatically increased at E 14 (~ 3 fold) and remained high until E 17. TGF- $\beta$ 2 gene expression sharply declined (~ 2.5 fold) and remained low until hatching. TGF- $\beta$ 2

mRNA from pectoralis muscles of *in ovo* rhIGF-I treated embryos dramatically increased at E 13 (~ 2.5 fold), in contrast to E 14 from control pectoralis muscle, and gradually declined through E 16. Additionally, TGF- $\beta$ 2 gene expression in the *in ovo* rhIGF-I injected group, unlike the control groups, showed a sudden increase at E 18 prior to hatching.

Figure 5 depicts the patterns of  $\beta$ -Actin gene expression in both control and *in ovo* rhIGF-I injected chicken embryos.  $\beta$ -Actin was used as a housekeeping gene and was stable throughout the experiment in both groups.

## **DISCUSSION**

Strong myostatin mRNA expression in chicken (Cobb X Cobb) was previously seen during the blastoderm stage of embryos and remained high through embryonic day 2 (Kocamis *et al.*, 1999), whereas the same expression pattern was not observed in Ross X Ross chicken embryos of the present study. Because the Ross X Ross strain is considered a fast growing chicken lines, the different myostatin expression pattern between these two strains could be due to the different growth rate of these birds. Therefore, early myostatin expression may not only determine muscle growth but also play a pivotal role in early embryonic growth, which ultimately gives rise to either faster or slower growing chicken lines. Nevertheless, this assumption needs to be further explored. Skeletal muscle tissue is widely believed to be the main source of myostatin (McPherron *et al.*, 1997). Embryonic chicken myoblasts begin to form in somites at approximately E 2.5 (for review, Stockdale *et al.*, 2000). It is, therefore, possible to postulate that early myostatin expression (E 4) in the present study originated from those presumptive myoblasts. However, this assumption does not preclude the possibility of myostatin

expression generated from other early chicken embryonic tissues, because myostatin mRNA was detected in adipose tissues and tubuloalveolar glands (Ji et al., 1998). Therefore, further studies using *in situ* hybridization should be conducted to verify the exact origin of myostatin gene expression in early chicken embryonic development.

Overall follistatin gene expression profile from control group supported our previous findings which demonstrated that follistatin mRNA level increased in a linear fashion from E 1 to E 20 (Kocamis *et al.*, 1999). A single *in ovo* rhIGF-I injection seemed to shift the overall follistatin gene expression in chicken embryos and subsequently in pectoralis muscle while having less pronounced effect on activin-B expression. Activin-B gene expression pattern from control embryos was similar to our previous findings which showed that overall activin-B mRNA expression followed a quadratic fashion throughout embryonic development (Kocamis *et al.*, 1999). Given the fact that most, if not all, of the biological actions of activins are neutralized by its binding to follistatin (Michel *et al.*, 1993), activin-B and follistatin gene expression from control groups should follow the parallel ontogeny during embryonic development, irrespective of tissue or whole embryo. However, this was not observed in the present study, perhaps due to broad inhibitory effect of follistatin with other TGF- $\beta$  family members such as bone morphogenic proteins.

It was shown that exogenous IGF-I stimulated differentiation of L6A1 myoblasts through a process that involves myf-5 and an increase in the level of myogenin expression (Florini et al., 1991). Also, exogenous IGF-I suppressed the IGF-II expression in those cell lines (Magri et al., 1994). For this reason, the influence of *in ovo* rhIGF-I on myogenic regulatory genes (myoD, myf-5, MRF-4 and myogenin) and IGF-

II gene expression during chicken embryonic muscle development is under investigation in our laboratory. In the current study TGF- $\beta$ 2, a well known representative member of the TGF- $\beta$  family, was chosen to determine whether the myostatin response to the *in ovo* administration of rhIGF-I was distinct from that of the other TGF- $\beta$  family members. As shown in figure 2, TGF- $\beta$ 2 gene expression, unlike myostatin, demonstrated late response (E 13 versus E 9) to the rhIGF-I. Bosche et al. (1995) demonstrated that each TGF- $\beta$  isoform investigated in L6A1 myoblast cell culture responded differently to the addition of IGF-I. For instance, TGF- $\beta$ 2 was less responsive than TGF- $\beta$ 1 and TGF- $\beta$ 3. These findings could, to some extent, explain the varying response of myostatin, follistatin, activin-B, and TGF- $\beta$ 2 gene expression in the rhIGF-I treated group.

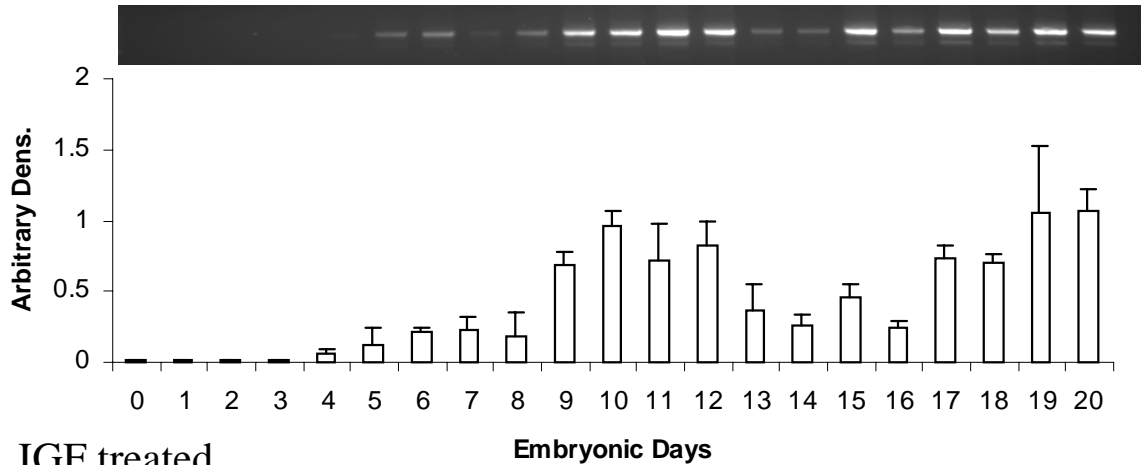
Even though growing pigs postnatally treated with porcine growth hormone (GH) showed ~ 35% increased muscle mass, myostatin mRNA abundance in skeletal muscle tissues of these animals was not affected (Ji et al., 1998). This hypertrophic growth was due predominantly to satellite cell proliferation and the incorporation of these nuclei into existing myofibers. Given the fact that GH exerts its mitogenic functions mainly through liver IGF-I production (for review, Florini et al., 1996), IGF-I stimulated satellite cell proliferation may not be due directly to an alteration of myostatin gene expression. Additionally, IGF-I gene expression, unlike myostatin, was not detected in satellite cells derived from either pectoralis major or biceps femoris muscles of chicken (Kocamis et al., 2001). Therefore, the physiological role of myostatin in muscle may mainly be associated with the prenatal period of muscle growth, which coincides with our findings of altered developmental myostatin expression pattern in response to *in ovo* rhIGF-I during embryonic development.

Although overall myostatin expression patterns in both control and rhIGF-I injected groups seem to be similar during embryonic pectoralis muscle development, *in ovo* administration of rhIGF-I on E 3 appeared to delay myostatin expression approximately one day compared to control embryos. A delay of myostatin expression would allow myoblasts additional time in the replicative cell cycle which would ultimately lead to increased muscle fiber formation. Thus, delayed expression of myostatin may be responsible for the muscle hyperplasia associated with postnatal chicken skeletal muscle increase due to *in ovo* rhIGF-I administration (Kocamis et al., 1998, 2000).

There is a delicate balance between autocrine and paracrine production of growth factors as well as endocrine production of hormones to ensure that a consistent number of muscle fibers are formed. Increased muscle mass in response to a single administration of *in ovo* rhIGF-I during early chicken embryonic development (Kocamis et al., 1998, 2000) might be due to a perturbation of this homeostatic balance of factors produced locally in the muscle. However, considering the complexity of the IGF system, such as secretion of binding proteins and presence of two surface receptors, it is difficult to pin-point the exact mechanism of a single *in ovo* rhIGF-I injection that altered developmental expression patterns of myostatin, follistatin, activin-B and TGF- $\beta$ 2 genes during chicken embryonic development. In conclusion, our results demonstrate that *in ovo* administration of rhIGF-I on E 3 alters developmental expression patterns of myostatin, follistatin, and activin-B and TGF- $\beta$ 2 genes. A complete understanding of the interaction between IGF-I and the TGF- $\beta$  family genes

during embryonic development could be beneficial to human health and food animal agriculture.

## Control



## IGF treated

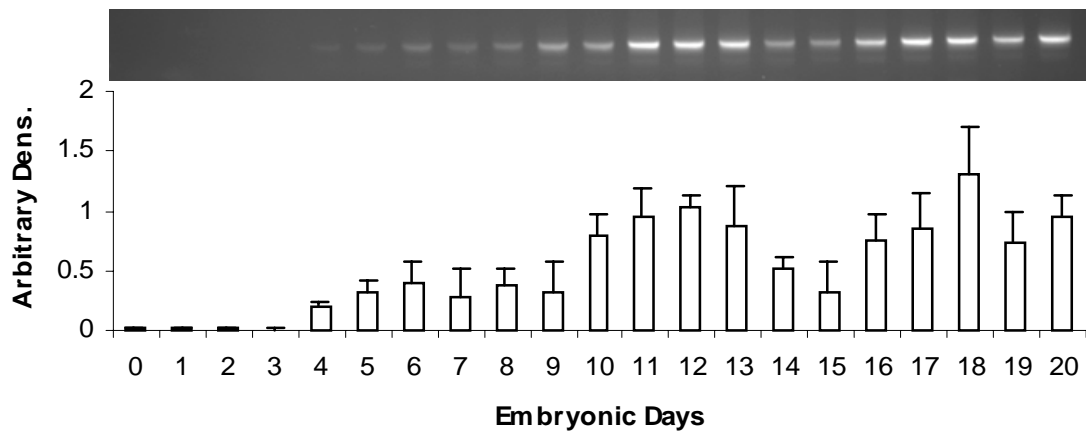


Figure 1. Representative steady-state levels of control and IGF-I treated group myostatin mRNAs in whole embryo and pectoralis muscle during chicken embryonic development (n= 6 or 4 per day, respectively). The bands for myostatin mRNA were analyzed by densitometry and the integration values (mean  $\pm$  SD), after normalization to  $\beta$ -actin, were expressed in arbitrary densitometric units at each sampling day.

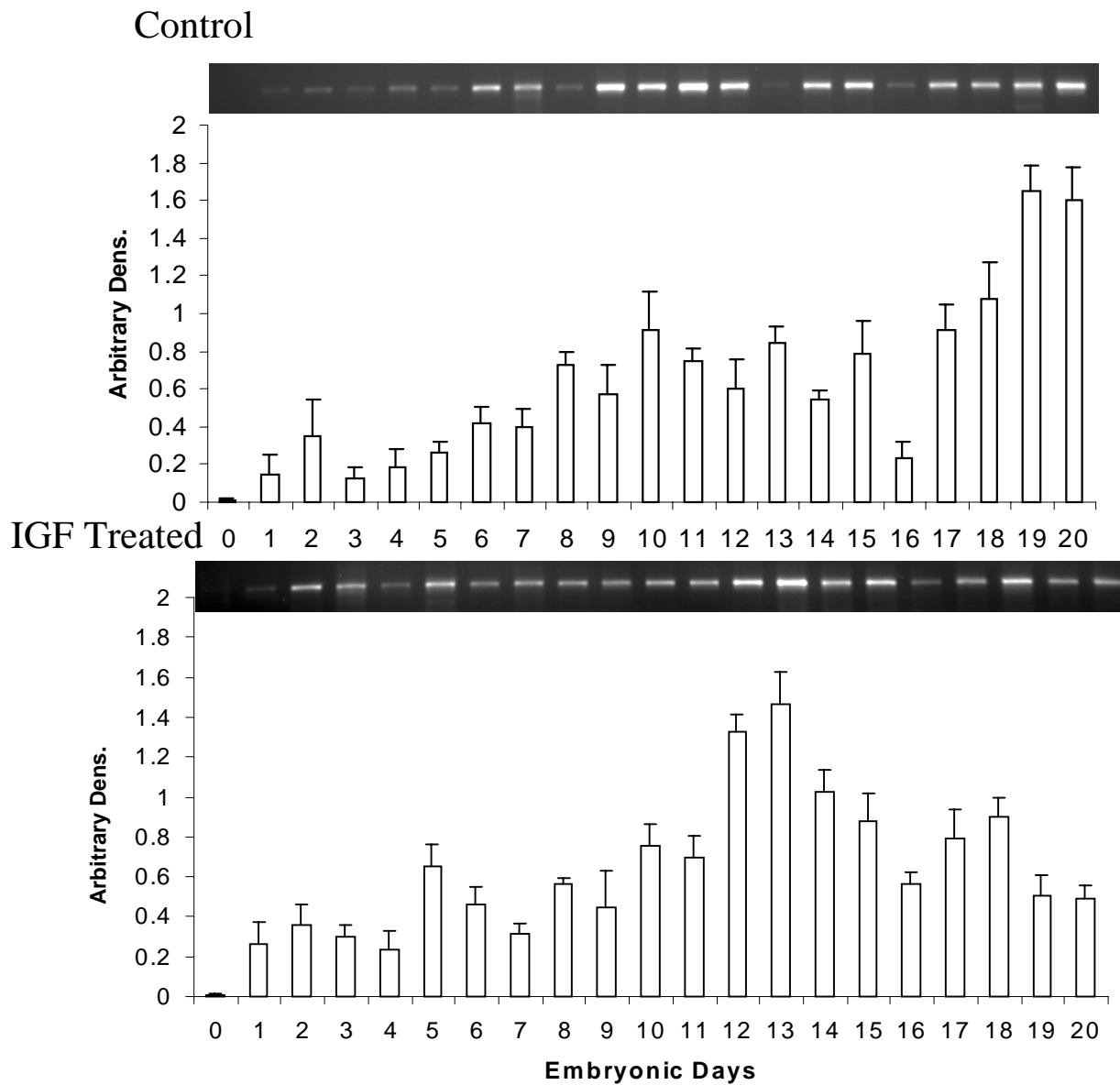


Figure 2 Representative steady-state levels of control and IGF-I treated group follistatin mRNAs in whole embryo and pectoralis muscle during chicken embryonic development (n= 6 or 4 per day, respectively). The bands for follistatin mRNA were analyzed by densitometry and the integration values (mean  $\pm$  SD), after normalization to  $\beta$ -actin, were expressed in arbitrary densitometric units at each sampling day.



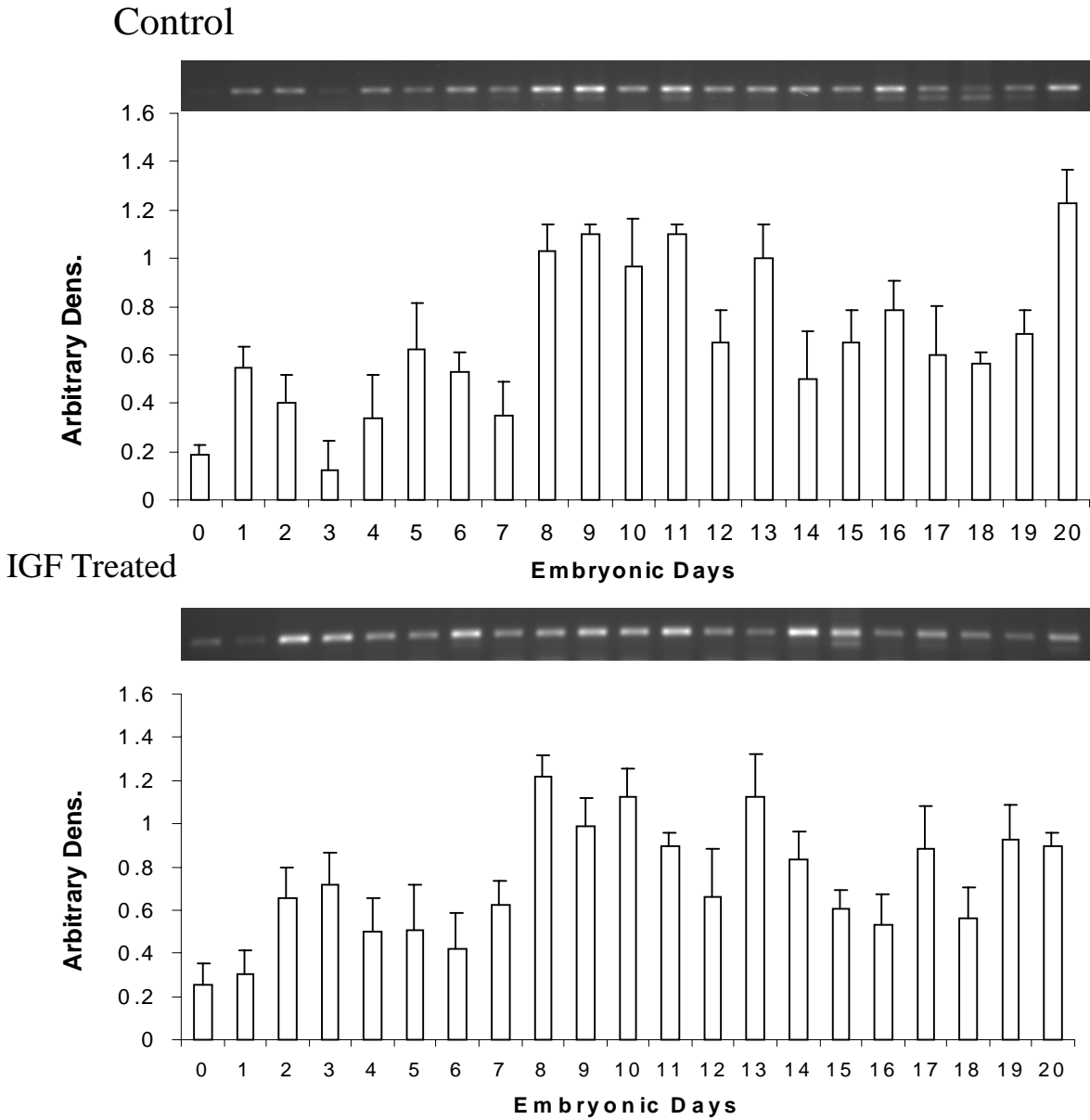


Figure 3 Representative steady-state levels of control and IGF-I treated group activin-B mRNAs in whole embryo and pectoralis muscle during chicken embryonic development (n= 6 or 4 per day, respectively). The bands for activin-B mRNA were analyzed by densitometry and the integration values (mean  $\pm$  SD), after normalization to  $\beta$ -actin, were expressed in arbitrary densitometric units at each sampling day.

## Control

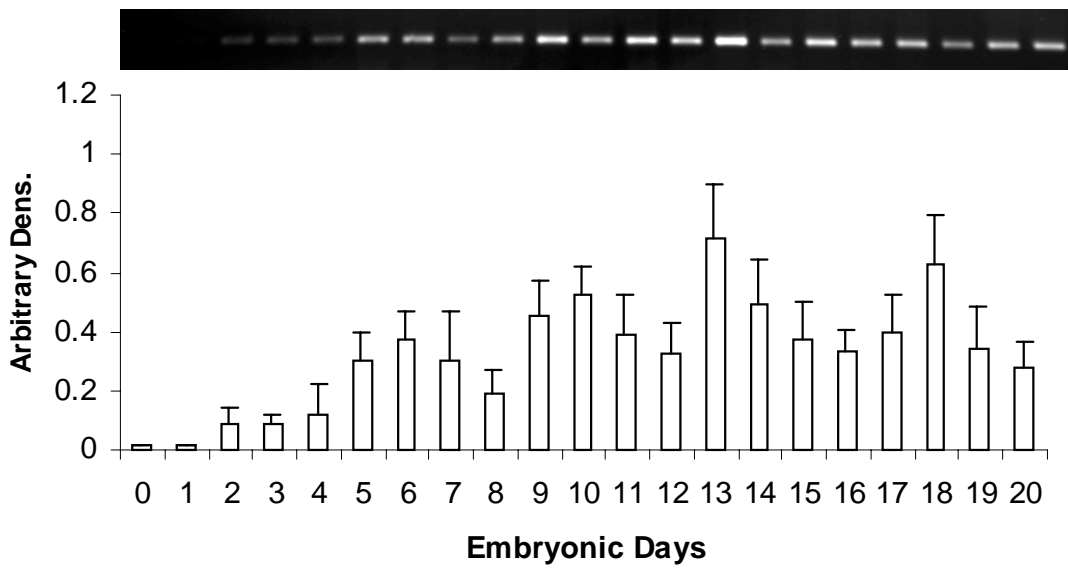
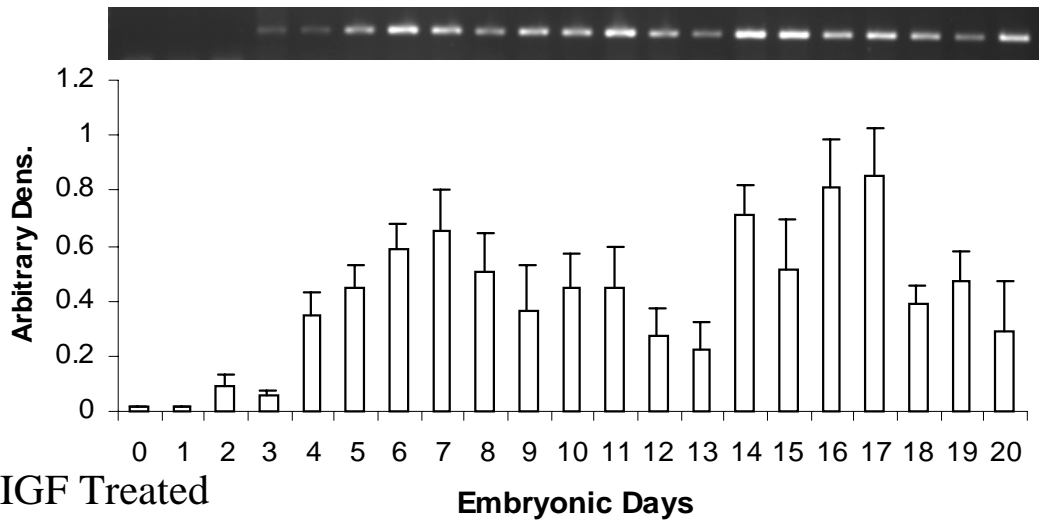


Figure 4 Representative steady-state levels of control and IGF-I treated group TGF- $\beta$ 2 mRNAs in whole embryo and pectoralis muscle during chicken embryonic development (n= 6 or 4 per day, respectively). The bands for TGF- $\beta$ 2 mRNA were analyzed by densitometry and the integration values (mean  $\pm$  SD), after normalization to  $\beta$ -actin, were expressed in arbitrary densitometric units at each sampling day.

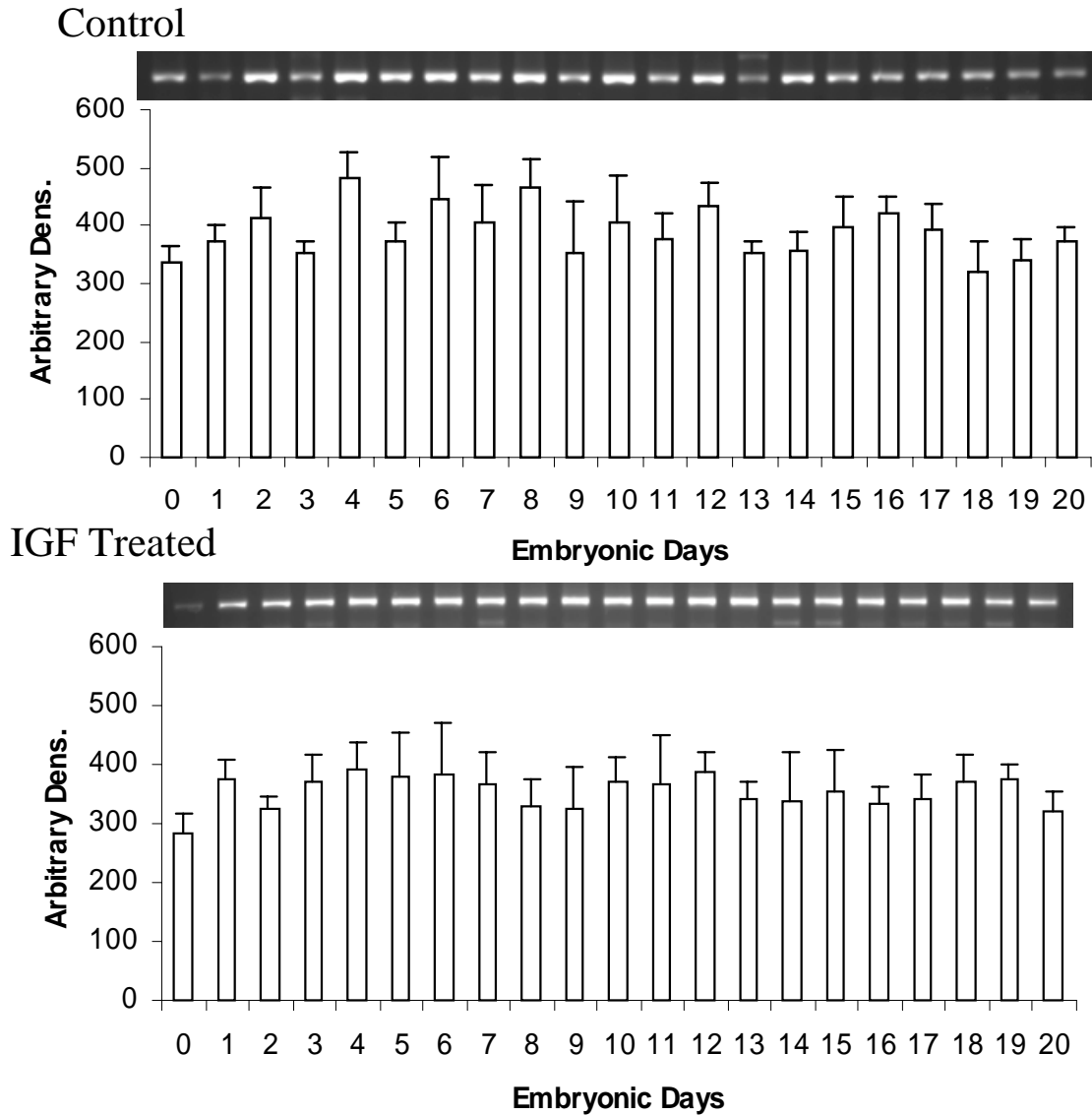


Figure 5 Representative steady-state levels of control and IGF-I treated group  $\beta$ -actin mRNA in whole embryo and pectoralis muscle during chicken embryonic development (n= 6 or 4 per day, respectively). The bands for  $\beta$ -actin mRNA were analyzed by densitometry and the integration values (mean  $\pm$  SD) were expressed in arbitrary densitometric units at each sampling day.

## **Chapter 4**

### **IGF-I, IGF-II AND IGF RECEPTOR-I TRANSCRIPT AND PROTEIN EXPRESSION IN MYOSTATIN KNOCKOUT MICE TISSUES**

## INTRODUCTION

Insulin-like growth factors (IGF-I and -II) are produced by several different tissues and are essential for both embryonic and postnatal development (Coolican et al., 1997, LeRoith, 1997). Severe disruption of tissue development, particularly in skeletal muscles, was observed in IGF-I or IGF-II knockout mice. For instance, IGF-I knockout mice were significantly smaller than their control littermates and had severe muscular dystrophy (Powell-Braxton et al., 1993), whereas transgenic mice overexpressing IGF-I showed enhanced body growth with an increase in muscle mass (Mathews et al., 1988). Additionally, IGF-II expression has been positively associated with skeletal muscle development in double-muscling cattle (Keller et al., 1999) and in pig breeds with exceptional muscle mass (Nezer et al., 1999). *In vitro*, both IGFs inhibited apoptosis (Wingertzahn et al., 1998) and promoted proliferation and differentiation of skeletal muscle cells (Bark et al., 1998). As evidenced by these *in vivo* and *in vitro* findings, IGFs are undoubtedly important components of skeletal muscle development.

When the myostatin gene (also known as growth differentiation factor/8), a recently identified member of the TGF- $\beta$  family, was disrupted by homologous recombination in mice, skeletal muscle mass significantly increased, up to the three times normal size (McPherron et al., 1997). Increased muscle mass in these mice was due predominantly to hyperplasia but also involved hypertrophy. Additionally, myostatin mutation resulting in functional loss of the protein has been linked to double-muscling cattle breeds (Grobert et al., 1997, Kambadur et al., 1997). Therefore, myostatin has been proposed to be a negative regulator of skeletal muscle growth. We postulated that the ratio of mRNA and protein levels between IGFs, positive regulators of muscle growth,

and myostatin, a negative regulator, may serve as indicators of skeletal muscle mass. Also, based on the gene knockout observations, IGFs, in addition to being a pivotal regulator of muscle growth, appear to be involved in controlling overall growth, whereas myostatin seems to be acting only on muscle growth. Thus, to demonstrate whether a correlation exists between IGFs and myostatin, IGF-I, -II and IGF receptor-I mRNA (IGF-R1) and protein levels were determined in a wide variety of myostatin knockout mice tissues.

## **MATERIALS and METHODS**

### **Animals**

Nine months old male myostatin knockout (n= 3) and control mice (n= 3, SVJ/129) were generously provided by Metamorphix Inc., (Baltimore, MD). Euthanasia of mice and subsequent tissue collections were performed in compliance with an approved West Virginia University Animal Care and Use Committee Protocol.

### **RNA extraction and RT-PCR**

Total RNA was extracted separately from myostatin knockout and control mouse brain, heart, liver, kidney, and skeletal muscle tissues (soleus, gastrocnemius, and pectoralis) using the Tri-Reagent (Sigma, St. Louis, MO) modification of the guanidine isothiocyanate/phenol-chloroform method (Chomczynski and Sacci, 1987). The RNA concentration was estimated by absorbance at 260 nm using a Shimadzu spectrophotometer (Columbia, MD). Samples of RNA were stored at -80 C.

Reverse transcription (RT) was performed as described in Chapter 1 and 2. The PCR reaction started with one cycle consisting of 94 C for 5 minutes, an annealing step of 65 C for IGF-I or 55 C for IGF-II and  $\beta$ -actin for 1 minute and extension at 72 C for 1

minute. The first cycle was followed by 30 cycles (25 cycles for  $\beta$ -actin) consisting of 45-sec intervals of 94 C, followed by 65 C for IGF-I or 55 C for IGF-II and  $\beta$ -actin, followed by 72 C. For IGF-RI, touchdown PCR was run. This program consisted of a 5-minute, 94 C denaturation step, followed by 5 cycles in which the initial annealing temperature of 72 was reduced by 1C per cycle, then 30 cycles in which the annealing temperature was 68C. Denaturation, extension and annealing time were programmed as described above. To establish a linear range of amplification for each gene, several different cycle numbers of PCR (10, 15, 20, 25 and 30 cycles) were run. As a control, a PCR reaction without c-DNA was run, and no contamination was found in the reaction mixtures (data not shown).

### **PCR primers**

All PCR primers were synthesized by Gibco BRL Inc. (Grand Island, NY). Primers for IGF-I were designed on the basis of published sequences of chicken IGF-I (Bell et al., 1986). The sequence of the forward primer was 5'GCTGAGCTGGTGGATGCTCTTCAGTTC3', while the reverse primer was 5'CTTCTGAGTCTTGGGCATGTCAGTGTG 3'. Forward and reverse primers predicted a PCR product of 215 base pairs (bp), which corresponds to bases (160-265) of the sequence. Primers for IGF-II were designed on the basis of published sequences of chicken IGF-II (Rotwein and Hall, 1990). Primers for IGF-II amplified a PCR product of 356 bp which corresponded to bases (1041-1397) of the sequence. The sequence of the forward primer for IGF-II was 5' GAGCTTGTTGACACGCTTCAGTTTGTC 3', while the reverse primer was 5' ACGTTTGGCCTCTCTGAACTCTTTGAG 3'. Primers for IGF-RI were designed on the based of published sequence of mouse IGF-I (Wada et al.,

1993). The sequence of the forward primer was 5' GACATCCGCAACGACTATCAG3', while the reverse primer was 5' GTAGTTATTGGACACCGCATC 3'. Primers for IGF-RI amplified a PCR product of 395 bp which corresponded to bases (114-509) of the sequence. Forward and reverse primers for  $\beta$ -actin were predicted to amplify a 285 bp product as previously published (Yamamura et al., 1991), as an internal standard to verify the level of amplification. The sequence of the forward primer was 5' TCATGAAGTGTGACGTTGACATCCGT 3', while the reverse primer was 5' CCTAGAAGCATTGCGGTGCACGATG 3'.

The amplified PCR products for each gene were visualized on 1.5 % agarose gels stained with ethidium bromide. Products were analyzed by densitometry of stained gels, and data should, therefore, be considered to be semi-quantitative. The identity of all PCR products was confirmed by sequence analysis.

### **Immunohistochemistry**

Kidney and soleus muscles from adult myostatin knockout and control mice were immersion fixed in Prefer fixative (Anatech, Battle Creek, MI). Sections were processed overnight and embedded in paraffin. Unstained sections were cut at 5 microns and incubated at 60°C for 20 minutes. Sections were then deparaffinized in 3 baths of xylene (6 minutes each), a bath of 100% alcohol (3 minutes), a bath of 95% alcohol (3 minutes), a bath of 80% alcohol (3 minutes) and a final bath of distilled water for 5 minutes. The sections were then placed in a coplin jar with 0.01M EDTA (pH = 8, Fisher Scientific, Pittsburgh, PA) for antigen retrieval. Coplin jar was placed in microwave on high for 1 minute, 45 seconds to bring temperature up to boiling. A defrost cycle was set for 6 minutes that kept the solution just below boiling. After 6 minutes, the coplin jar was



removed from the microwave and allowed to sit for 20 minutes. The slides were then rinsed and incubated in distilled water for 5 minutes. All procedures for immunochemistry were done in a Humidity Chamber (Shandon Lipshaw, Pittsburgh, PA). Slides were incubated in 3% hydrogen peroxide for 10 minutes at room temperature. After rinsing with distilled water, slides were incubated in Tissue Conditioner (Biomed, Foster City, CA) for 10 minutes at 37° C (no humidity chamber). Slides were rinsed with distilled water and placed in TBS (Dako, Carpinteria, CA) for 5 minutes. After diluting with antibody diluent (1/100, Dako, Carpinteria, CA), IGF-II goat anti human antibody (Sigma, St. Louis, MO) was applied as a drop on section for 1 hour at room temperature. Slides were then rinsed with distilled water and incubated in TBS for 5 minutes at room temperature. Vectastain biotinylated secondary antibody (anti goat IgG) was applied as outlined by manufacturer (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) for 30 minutes at RT. Slides were then rinsed with distilled water and incubated in TBS for 5 minutes at room temperature. Vectastain ABC (avidin-biotinylated enzyme complex) reagent was applied for 30 minutes at RT. Slides were rinsed with distilled water and incubated in TBS for 5 minutes at room temperature. Aqueous Hematoxylin (Biomed, Foster City, CA) was applied as a drop on over section for 2 minutes to counterstain. Then slides were rinsed in tap water and coverslipped with Crystal Mount (Biomed, Foster City, CA). After crystal mount was dried, they were post mounted with Permount (Fisher Scientific, Pittsburgh, PA) and a glass coverslip. Whole section digital images were captured using a Sprint Scan slide scanner. Digital photomicrographs were captured using a Quantix digital camera. For digital capture, images from control and knockout mice were captured in the same session using identical settings. As described in appendix

D, western blotting was used to validate the quality of the primary antibody. Rabbit Super Sensitive Control Serum (Biogenex, San Ramon, CA) in place of primary antibody was used as a negative control on one section for each run.

### **Statistical Analysis**

PDIF of LS means was used to compare means (GLM procedure of SAS<sup>®</sup>, SAS Institute, 1989).

## **RESULTS**

### **$\beta$ -Actin, IGF-I, IGF-II and IGF-R1 mRNA Expression**

$\beta$ -Actin mRNA expression was not different for the same tissues between control and myostatin knockout mice (Fig. 1). IGF-I gene expression for brain, heart, liver, kidney and pectoralis muscles were similar between control and knockout mice, while no IGF-I mRNA was detected in either control or knockout mice soleus muscles (Fig. 2). IGF-II mRNA levels were significantly higher in myostatin knockout mice kidney and soleus muscles than that of control mice ( $P < 0.01$ , Fig. 3). No IGF-II gene expression was observed in liver and kidney of control mice, but only in liver of myostatin knockout mice (Fig. 3). IGF-Receptor-1 mRNA levels from control mice heart ( $P < 0.05$ ) and kidney ( $P < 0.01$ ) were significantly higher than that of myostatin knockout mice, while levels were lower in control mice pectoralis muscle than that of knockout mice ( $P < 0.01$ , Fig. 4). IGF-R1 gene expression was similar in brain, liver, soleus and gastrocnemius muscles of both control and myostatin knockout mice.

### **IGF-II Immunohistochemistry**

An IGF-II immunohistochemistry negative control is shown in figure 5. IGF-II was principally localized to small cells located adjacent to soleus muscle myofibers (Fig. 6).

The strongly IGF-II positive cells were common in myostatin knockout mice and were seen in a few foci in control mice. The IGF-II positive cells are believed to be satellite cells based on location and size. IGF-II immunoreactivity in both control and myostatin knockout mice kidneys was localized to the epithelium of renal tubules and collecting ducts (Fig. 7). No consistent differences in expression were noted between the two groups of mice.

## **DISCUSSION**

Although both IGF-I and -II are well known positive regulators of muscle growth, their gene expressions in the current study demonstrated different patterns between myostatin knockout and control mice muscle tissues. IGF-I mRNA levels from soleus (predominantly red fibers), gastrocnemius (predominantly white fibers) and pectoralis (white and red fibers) muscles were not different between myostatin knockout and control mice (Fig. 2), while IGF-R1 mRNA levels from pectoralis muscles were significantly higher in myostatin knockout mice than that of control mice (Fig 4). On the other hand, IGF-II mRNA levels from soleus muscles were higher in myostatin knockout mice than that of control mice, while no difference was observed between myostatin knockout and control mice gastrocnemius and pectoralis muscles (Fig. 3). Based on the immunohistological findings of the present study, elevated IGF-II in the soleus of myostatin knockout mice is believed to originate from strong expression of the IGF-II gene in activated satellite cells. Varying expression patterns of IGF-I and IGF-II in myostatin knockout mice soleus and gastrocnemius muscle could be explained as follows. First, because myostatin mRNA and protein levels were higher in gastrocnemius muscle (predominantly white fibers) than soleus muscle (predominantly red fibers) of

normal adult mice (Carlson et al., 1999), complete absence of myostatin in the knockout mice may have triggered distinct and novel muscle regulatory pathways in these two different fiber type dominated muscles, thereby causing the observed differences in the expression patterns of IGF-I and -II. Second, Semsarian et al. (1999) demonstrated that myostatin mRNA level was not affected by IGF-I overexpression in C2C12 cell cultures. Along the same line, the loss of skeletal muscle mass induced during space flight of rats was associated with increased myostatin mRNA and protein levels in the skeletal muscle and decreased IGF-II mRNA, while no change in IGF-I mRNA level was observed in those rats (Lalani et al., 2000). Based upon these *in vitro* and *in vivo* findings, it is thus possible to expect that complete absence of myostatin should increase IGF-II mRNA levels without effecting IGF-I levels. Indeed, our finding of strong IGF-II mRNA and protein expression in soleus muscle of myostatin knockout mice and no difference in IGF-I mRNA levels of soleus, gastrocnemius and pectoralis muscle between these mice partially supports the *in vitro* and *in vivo* findings. Even though no changes in either IGF-I or IGF-II levels from pectoralis muscles were observed between myostatin knockout and control mice, IGF-R1 expression from the same muscle was significantly increased in myostatin knockout mice (Fig. 4). Because both IGFs use IGF-R1 for their mitogenic and myogenic signal transduction pathways (for further review see, Florini et al., 1996), increased IGF-R1 expression without corresponding increases of ligands from pectoralis muscles of myostatin knockout mice remains to be further explored.

In the present study, strong IGF-II gene expression was observed in myostatin knockout mice kidney, while IGF-R1 expression was significantly lowered (Fig. 3 and 4, respectively). However, no consistent differences in IGF-II immunoreactivity were

observed between the two groups of mice. This was not totally unexpected, since the enzymatic immunohistochemical techniques use amplification cascades designed to maximize chromagen precipitation resulting from low levels of antibody binding. Also, the similar distribution of IGF-II in control and knockout mice (Fig. 7) suggests that changes in IGF-II expression result from increased expression in cells that normally express IGF-II rather than changes in the type of cells expressing IGF-II. Body weight of transgenic mice overexpressing IGF-II was not different from control mice but had increased kidney weight without any change in other organs (Wolf et al., 1994). Although myostatin knockout kidney weight was not measured in the current and previous studies (McPherron et al., 1997), increased IGF-II levels may have caused the decline in IGF-R1 mRNA expression to maintain proper size of myostatin knockout mice kidney. On the other hand, several studies suggested that metabolic actions of IGF-II, unlike its mitogenic actions, were not mediated through IGF-R1 but through insulin receptors, to which IGF-II can bind with low affinity (Czech, 1989, Hartmann et al., 1992). Additionally, IGF-II, but neither insulin nor IGF-I, stimulated  $\text{Na}^+\text{-H}^+$  exchange across the brush-border membrane of proximal tubular cells (for further review, see O'Dell and Day, 1998). Therefore, it is possible to postulate that increased IGF-II levels from myostatin knockout mice kidneys may be due to the disturbed metabolic balance in response to excess muscle growth of those mice. Further studies regarding the measurements of both metabolic parameters such as, blood glucose, fatty acids, and hormones such as insulin, growth hormone should be conducted to clarify this speculation.

Because of the fact that tissue samples were obtained only at one time point of age, we do not know whether the changes in IGF-I, IGF-II and IGF-R1 gene and protein expression were the same throughout the neonatal and adult life of the mice. Reciprocal changes in the expression of myostatin and IGF-II and IGF-R1 may facilitate the better understanding of not only skeletal muscle growth but also other organ development in mammals as well as pathophysiology of these tissues associated with disease.

## **SUMMARY**

During chicken embryonic development, the growth of each tissue is regulated by a very specific progression in the expression of genes which ultimately give rise to a viable chicken. In first two studies of this dissertation, the developmental patterns of genes that regulate skeletal muscle growth during embryonic development as well as satellite cells that are responsible for postnatal skeletal muscle growth were established. For instance, the ontogeny of myostatin gene expression in chicken embryos concurred with time frames of primary and secondary muscle fiber formation. Also, strong myostatin gene expression was found in the early chicken Cobb X Cobb embryos (E 0, E 1), suggesting that myostatin had a pivotal role during early chicken embryo development, before myogenic identity was established. However, myostatin gene expression was not detected until embryonic day 4 in Ross X Ross chicken embryos (fast growing chicken strain, study 3), as opposed to Cobb X Cobb embryos (slow growing chicken strain, study 1). These findings raise a question of whether myostatin has differential role in early development in either faster or slower growing chicken lines, even before myogenic identity is established. On the other hand, follistatin and activin-B genes followed the same expression pattern between Ross X Ross and Cobb X Cobb chicken embryos. Even though their expression patterns coincided partially with the major myogenic events taking place during chicken embryonic development (see appendix A), follistatin mRNA expression, an inhibitor of activin-B, did not exactly follow in parallel fashion with activin-B during either Ross X Ross or Cobb X Cobb

chicken embryo development. Based on these expression patterns, it is concluded that follistatin actions were not simply reciprocal to those of activin-B.

Myostatin expression was almost identical in satellite cells isolated from chicken pectoralis muscle (predominantly white fiber) and biceps femoris muscle (predominantly red fibers), which contradicted some previous findings. As mentioned earlier, there may be either species specific myostatin expression patterns in the two different types of muscles or alternate posttranslational modifications of the myostatin protein occurring in these muscles. Activin-B and follistatin expression followed parallel patterns in pectoralis major satellite cells, whereas the same expression pattern was not observed in biceps femoris satellite cells. Once again, these findings support the concept that follistatin may interact with other member of the TGF- $\beta$  family or other activin isoforms.

*In ovo* administration of rhIGF-I at embryonic day 3 resulted in enhanced skeletal muscle growth and feed efficiency of 42-day-old broiler chickens (Kocamis et al., 1998, 2000). For a second study, we attempted to determine whether a single *in ovo* administration of rhIGF-I at embryonic day 3 modified expression patterns of the aforementioned genes resulting enhanced muscle growth and feed efficiency. As detailed in Chapter 3, all the genes (myostatin, activin-B, follistatin, and TGF- $\beta$ 2) expression patterns were, to various degrees, affected by rhIGF-I administration. Given the complexity of the IGF system in chickens, it is difficult to identify the exact mechanism of how these changes took place in response to a single rhIGF-I injection.

The last experiment of this dissertation was conducted in myostatin knockout mice tissues to determine whether a correlation exists between IGFs, positive regulators of growth, and myostatin, a negative regulator of muscle growth. As detailed in Chapter



4, IGF-I mRNA levels from myostatin knockout mice tissues were not different from that of control mice. On the other hand, IGF-II mRNA levels from myostatin knockout kidney and soleus muscles were significantly higher than that of control mice. These findings lead us to reevaluate the concept of IGF-II involvement in growth and development of vertebrates. First, as opposed to general belief (Baker et al., 1993), growth-promoting actions of IGF-II in the mouse are not certainly restricted to embryogenesis. Secondly, IGF-II, instead of IGF-I, may, at least in red muscles, be the major regulator of satellite cell proliferation and differentiation during postnatal growth. Although these studies were conducted with two different species, this assumption was partially supported by our findings of strong IGF-II mRNA expression in chicken satellite cells isolated from two different muscles, while no IGF-I gene expression was detected in either cell line. Then, changes in gene expression patterns in response to a single *in ovo* rhIGF-I injection, as detailed in Chapter 3, could be due to increased IGF-II levels in those embryos. Given the fact that IGF-II specific receptors has not been found in chickens, IGF-I and IGF-II use the same IGF-I type 1 receptor for their mitogenic actions. Therefore, the impact of *in ovo* IGF-II administration during chicken embryonic development could be of interest.

Myostatin knockout mice showed a two- to three-fold increase in muscle mass when compared to control, without displaying any defects in function or morphology in skeletal muscle. Myostatin expression was inversely correlated with chronic muscle wasting illness in humans. Thus, myostatin may impair skeletal muscle repair by inhibiting cell replication or muscle fiber growth. It would be of interest to know whether myostatin is required for efficient regeneration of skeletal muscles. Additionally, identification of myostatin receptor (s), and its interaction with other TGF- $\beta$  family

receptors would benefit a better understanding of myostatin function under normal and diseased conditions. Furthermore, because our ultimate goal is to have more muscular, faster-growing and leaner chickens, any attempt to modify the patterns of these muscle specific gene expressions using various genetic techniques such as antisense oligonucleotides or their potential inhibitors (follistatin for myostatin) to enhance muscle growth and feed efficiency would tremendously improve the poultry and meat industry.

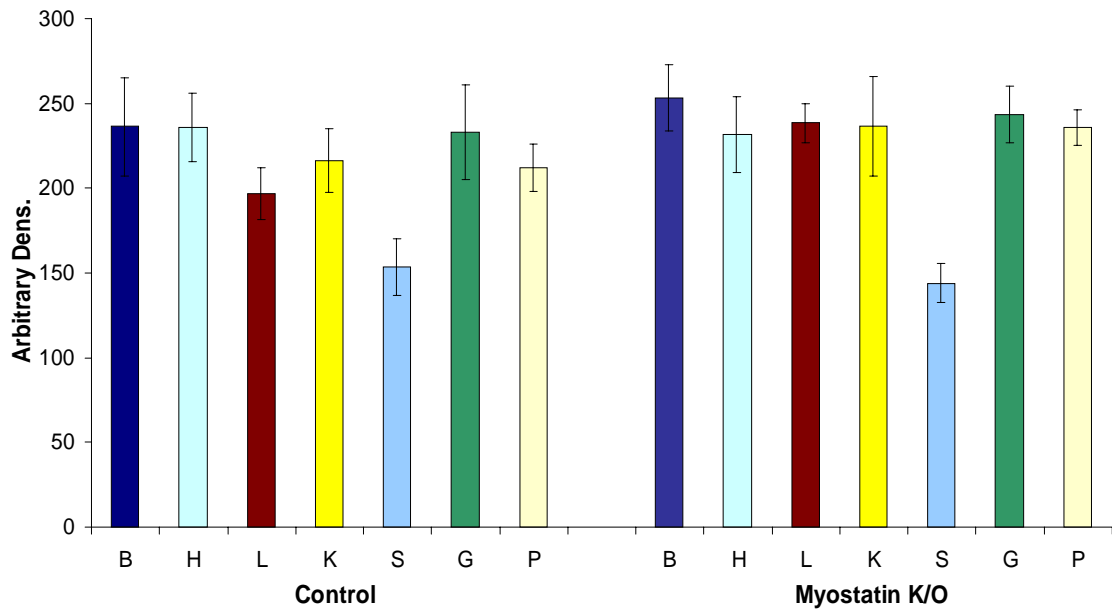
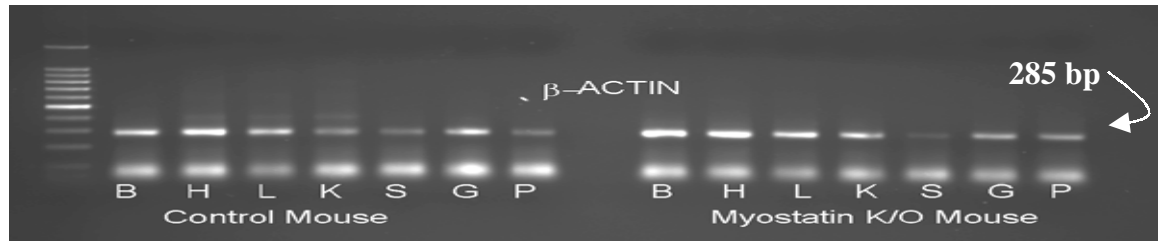


Figure 1. Steady-state levels of  $\beta$ -actin mRNA in brain (B), heart (H), liver (L), kidney (K), soleus (S), gastrocnemius (G), and pectoralis (P) muscle of control (n= 3) and myostatin knockout mice (n= 3) tissues. The bands for  $\beta$ -actin were analyzed by densitometry and the integration values (mean  $\pm$  SD) were expressed in arbitrary units for each tissue.

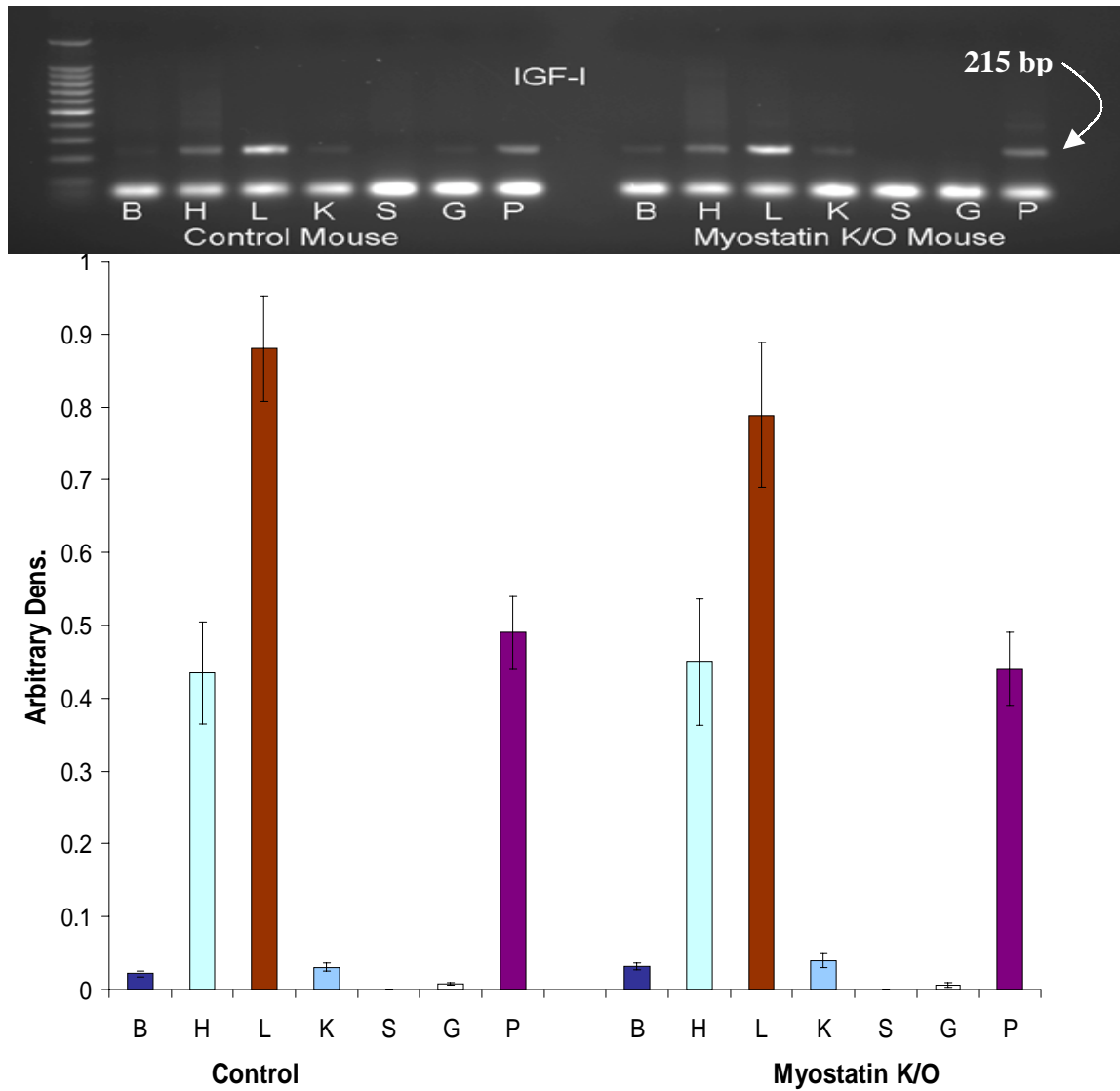


Figure 2. Steady-state levels of IGF-I mRNA in brain (B), heart (H), liver (L), kidney (K), soleus (S), gastrocnemius (G), and pectoralis (P) muscle of control (n= 3) and myostatin knockout mice (n= 3) tissues. The bands for IGF-I were analyzed by densitometry and the integration values (mean  $\pm$  SD), after normalization to  $\beta$  actin, were expressed in arbitrary units for each tissue.

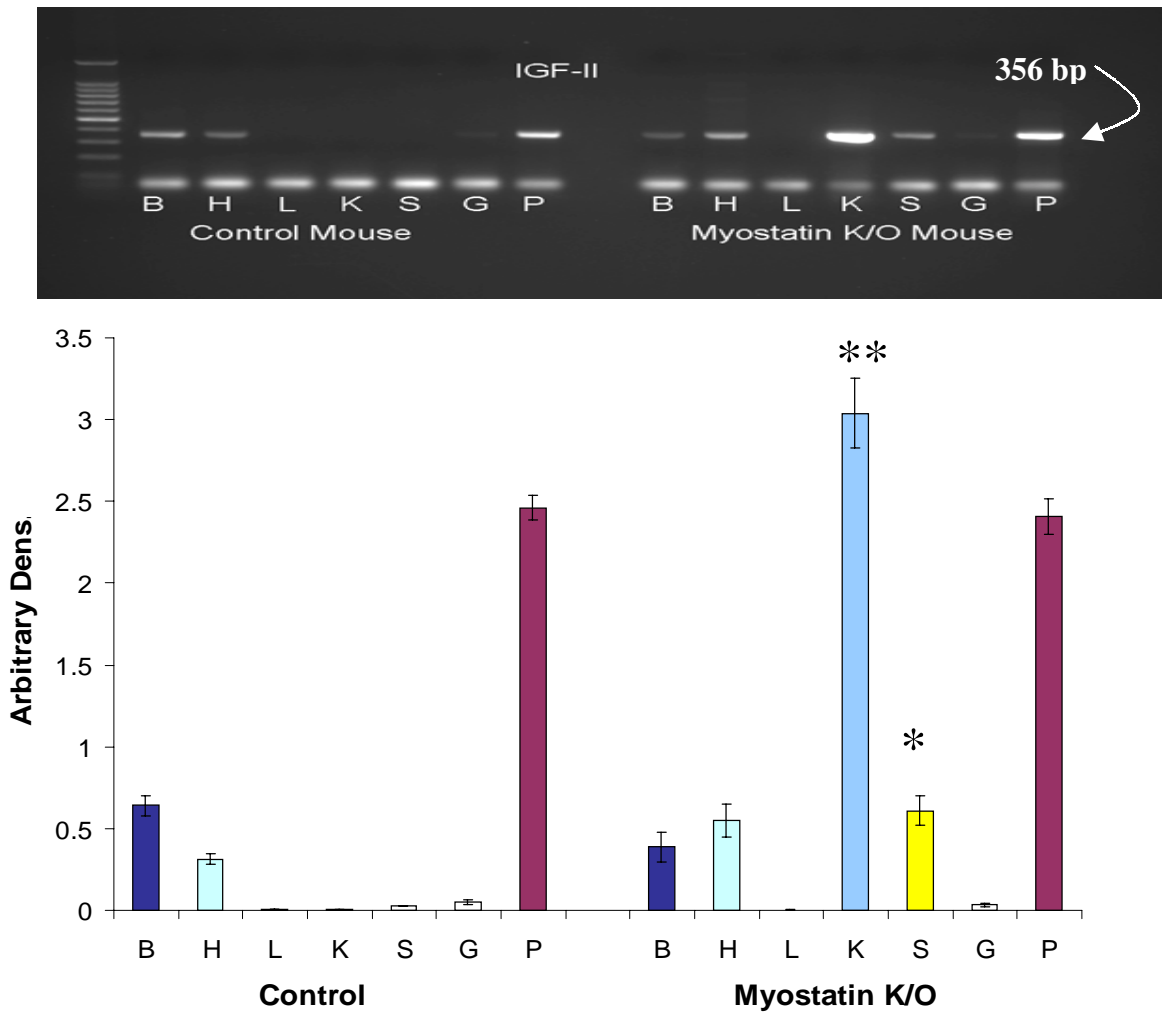


Figure 3. Steady-state levels of IGF-II mRNA in brain (B), heart (H), liver (L), kidney (K), soleus (S), gastrocnemius (G), and pectoralis (P) muscle of control (n= 3) and myostatin knockout mice (n= 3) tissues. The bands for IGF-II were analyzed by densitometry and the integration values (mean  $\pm$  SD), after normalization to  $\beta$  actin, were expressed in arbitrary units for each tissue. Means with asterisks were significantly different (\*\* P < 0.01).

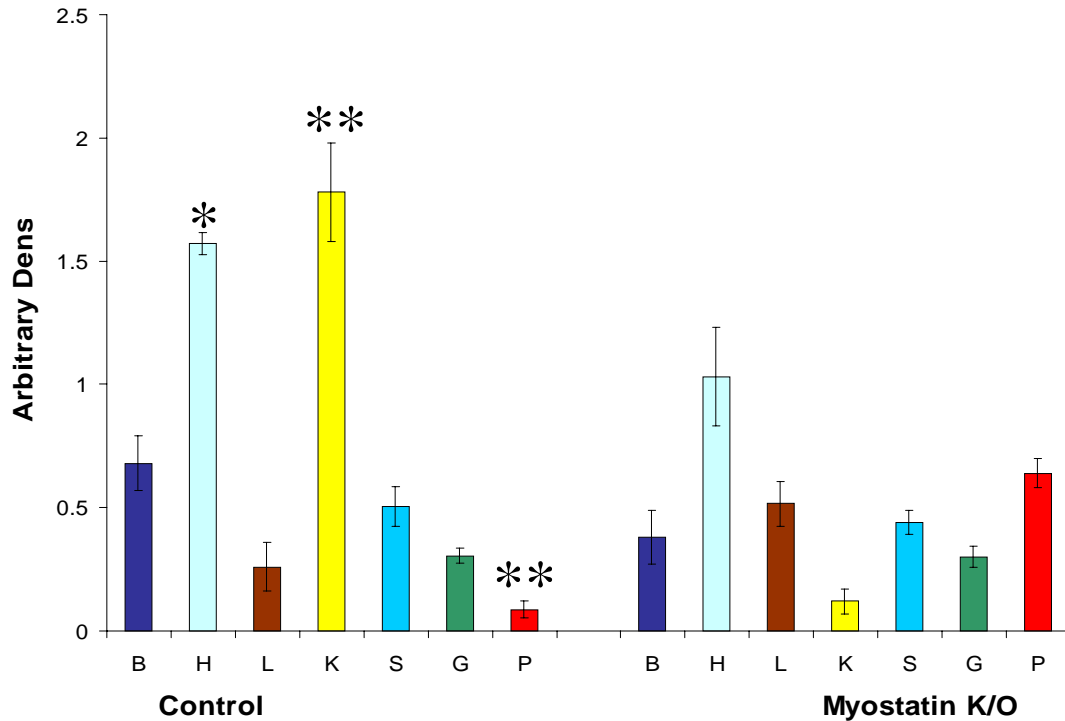
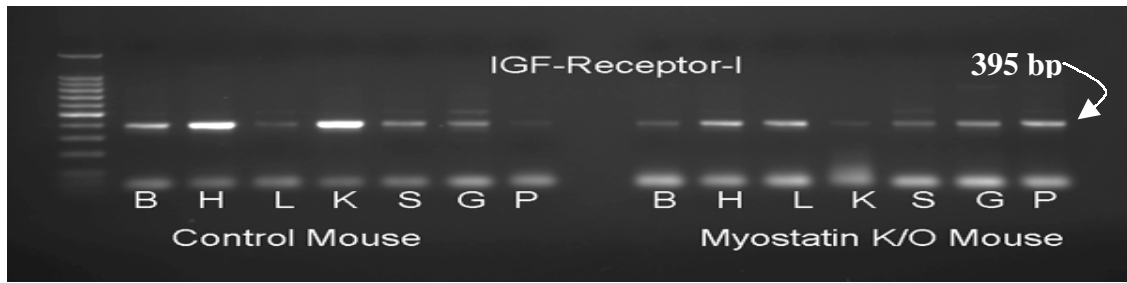


Figure 4. Steady-state levels of IGF-R1 mRNA in brain (B), heart (H), liver (L), kidney (K), soleus (S), gastrocnemius (G), and pectoralis (P) muscle of control (n= 3) and myostatin knockout mice (n= 3) tissues. The bands for IGF-R1 were analyzed by densitometry and the integration values (mean  $\pm$  SD), after normalization to  $\beta$  actin, were expressed in arbitrary units for each tissue. Means with asterisks were significantly different (\* P < 0.05, \*\* P < 0.01).

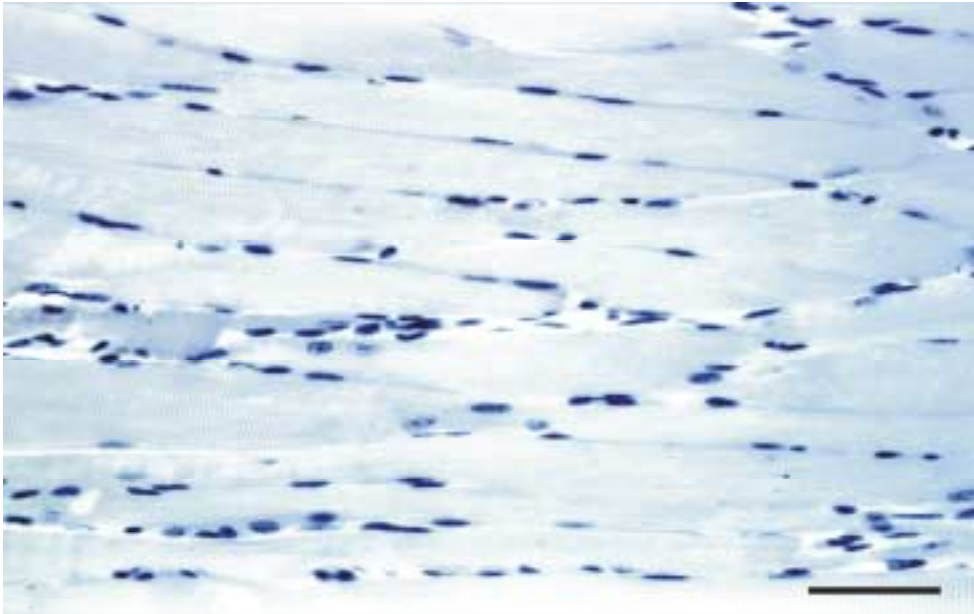
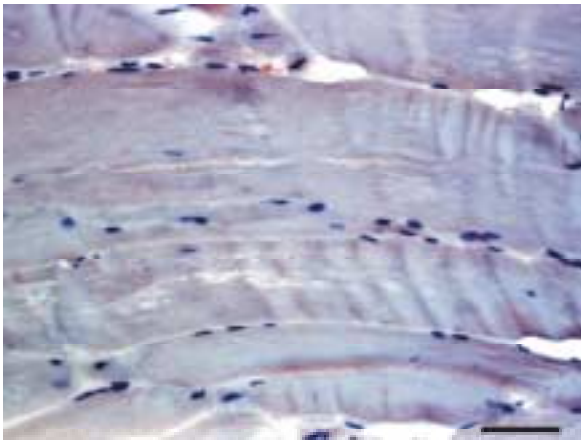
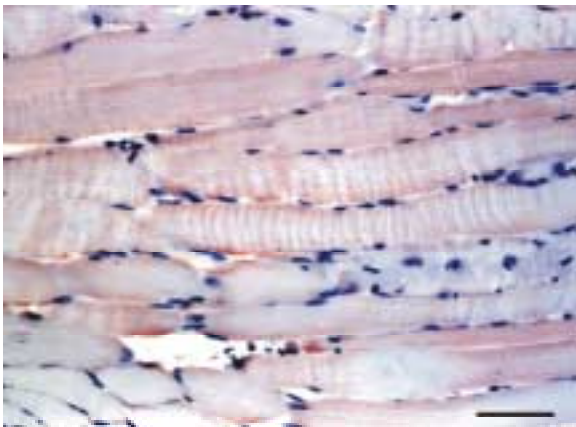


Figure 5. Rabbit Super Sensitive Control Serum (Biogenex, San Ramon, CA) in place of primary antibody was used as a negative control on one section for each run.

Control



Myostatin knockout

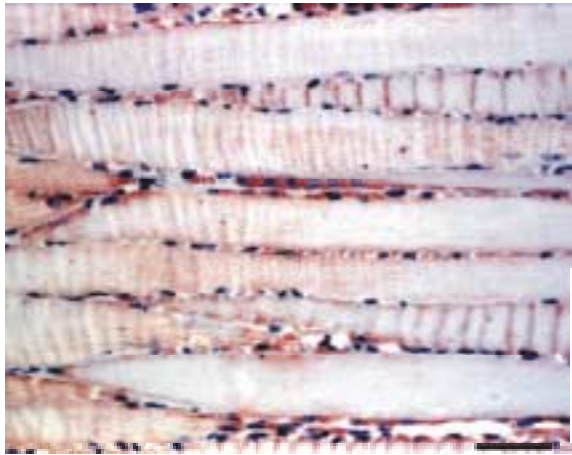
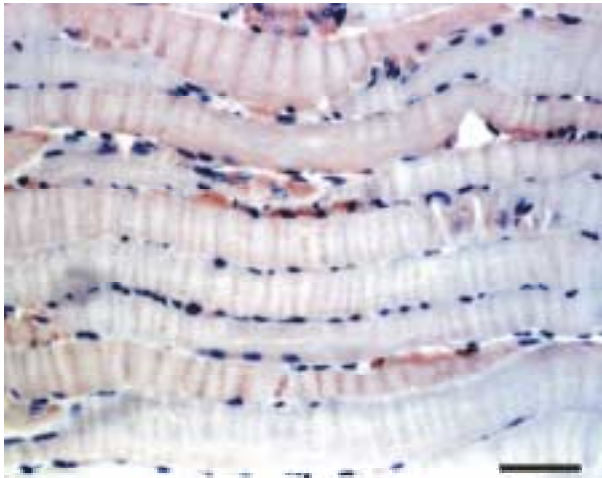


Figure 6. IGF-II immunohistochemistry in control and myostatin knockout mice soleus muscles. IGF-II immunoreactivity (blue dots) was localized satellite cells.



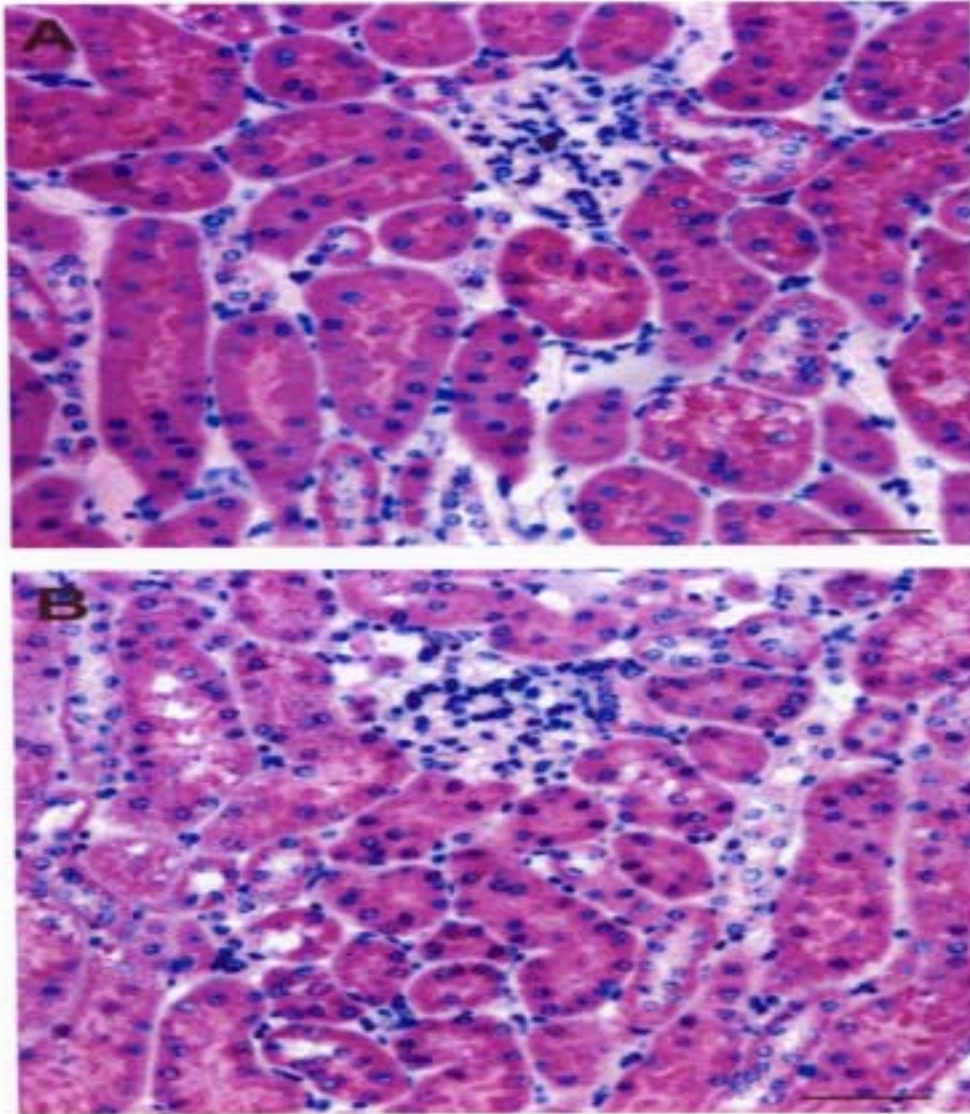


Figure 7. IGF-II immunohistochemistry in control (A) and myostatin knockout mice (B) kidneys. IGF-II immunoreactivity (blue dots) was localized to the epithelium of renal tubules and collecting ducts.

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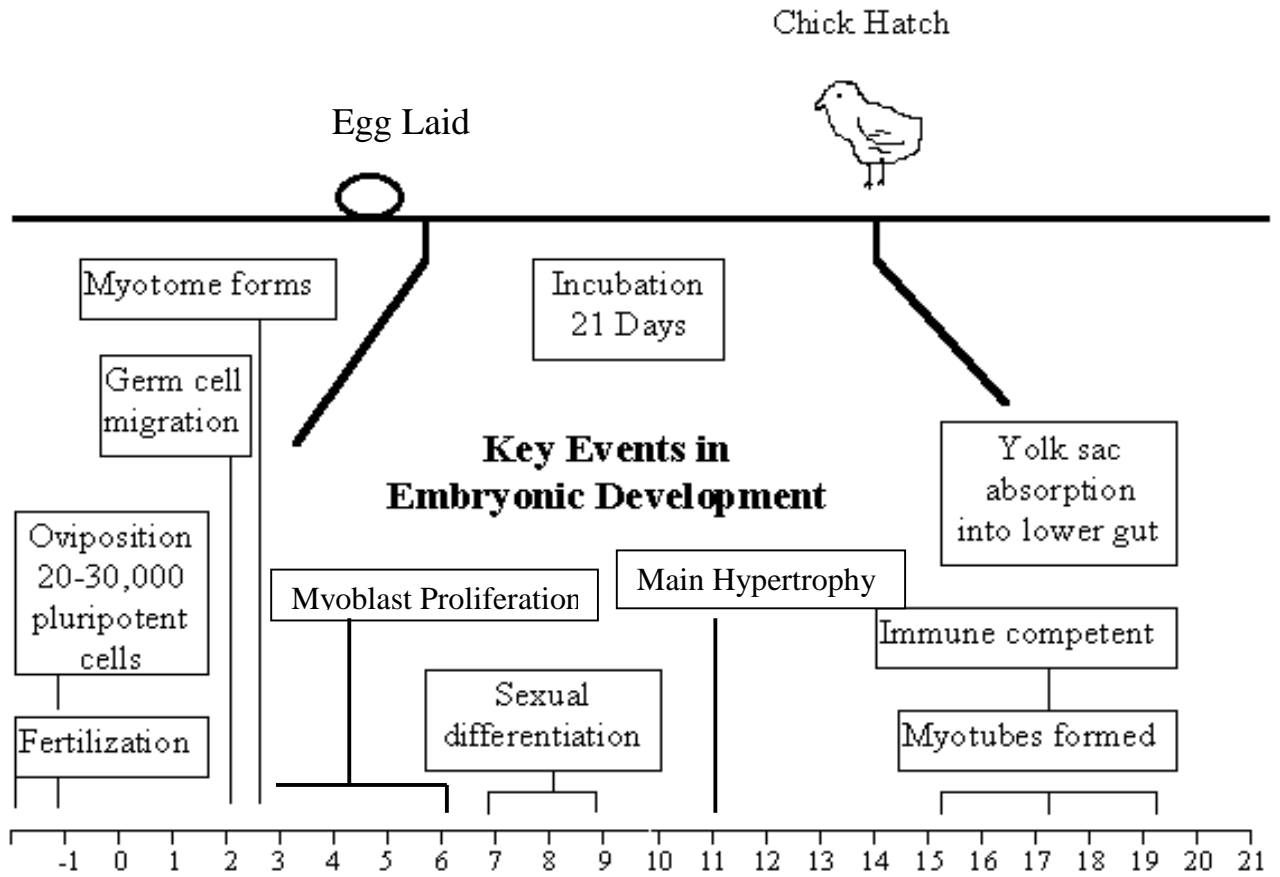


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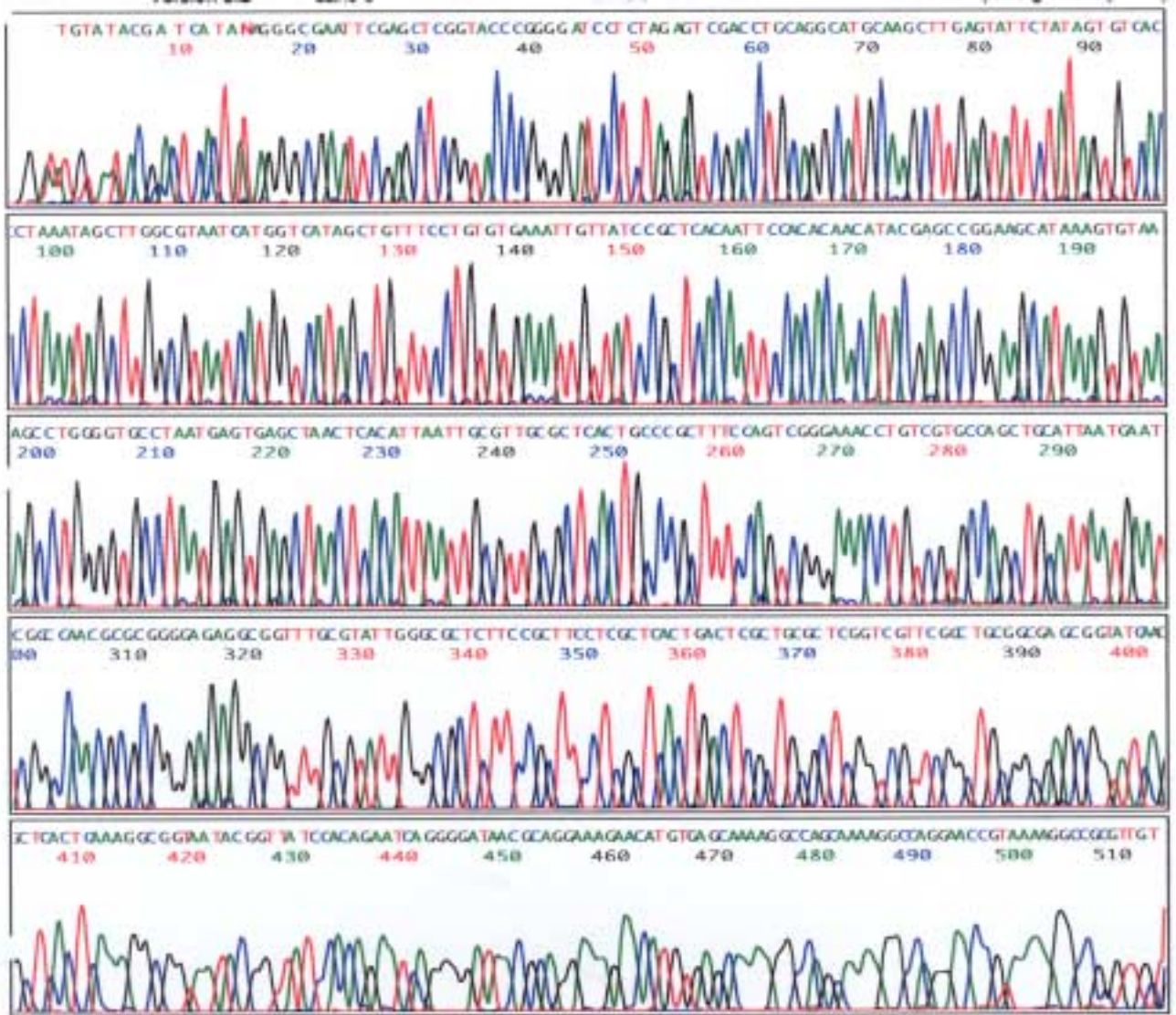
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APPENDIX

A)



B)



Chromatographic presentation of the myostatin RT-PCR product sequence.

Sequencing of RT-PCR products was performed as follows: First, PCR products were cut from gels and purified using Quantum Prep Freeze'N Squeeze DNA Gel extraction Spin Columns as outlined by the manufacturer (Bio-Rad, Hercules, CA). Second, purified PCR products were ethanol precipitated and quantified against  $\lambda$ /Hind3 mass marker (Promega, Madison, WI). A pGEM-T kit was used for ligation and

transformation reactions as described by the manufacturer (Promega, Madison, WI).

After overnight growth of individual colonies, plasmid preparation was conducted using a

QIA prep Spin Miniprep kit as outlined by the manufacturer (Qiagen, Valencia, CA).

Gene inserts were sequenced on an ABI Prism 310 Genetic Analyzer (Perkin-Elmer,

Foster City, CA).

C)



*In ovo* injection procedure.

D)

### **Western Blotting**

Tissues from myostatin knockout mice and control mice were homogenized in 1 volume of TE (100 mM Tris, and 10 mM EDTA, pH 8.3). Subsequently, homogenates were centrifuged at 4,000 X g for 5 minutes at 4 C. Supernatants containing proteins were mixed with 2X sample loading buffer (125 mM Tris, 4% SDS, 20% glycerol, 10% mercaptoethanol, 0.25% bromophenolblue, pH 6.8) and proteins were separated by SDS-PAGE (120 Volt for 1.5 h., 3.75% stacking, 12% separating gels). Then, proteins were electrophoretically transferred to nitrocellulose membrane (0.2  $\mu$ m, Protran, Keene, NH) for 1 hour at 4 C and 100 mA in buffer containing (25 mM) Tris, (192 mM) glycine, and 20% methanol in a Transblotter (Bio-Rad, Hercules, CA). Nitrocellulose membranes were rinsed in TBS (40 mM Tris, 0.15 M NaCl, pH 7.5) for 20 minutes and then blocked with 10% non-fat milk/TBS for 1 hour at room temperature. Membranes were then incubated with a 1:500 dilution (as recommended by manufacturer, Sigma, St Lois, MO) of either anti-human polyclonal insulin-like growth factor-II, insulin-like growth factor-I receptor or insulin-like growth factor-I (Fitzgerald, Concord, MA) in 10% non-fat milk/TBS at 4 C overnight. After washing three times for 10 minutes in 0.1% Tween 20 solution in TBS, membranes were incubated with a 1:30,000 dilution of anti-goat IgG-horseradish peroxidase conjugate in 10% non-fat milk/TBS for 1 hour at room temperature. Membranes were washed four times with 0.1% Tween/TBS solution (10 minutes each). After incubation with enhanced chemiluminescent substrate (Pierce, Rockford, IL), antigen-antibody complexes were detected by an image analyzer (Alpha Innotech, San Leandro, CA).