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Interaction between positive and negative effectors of embryonic and postnatal muscle growth in the chicken and the mouse

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INTERACTION BETWEEN POSITIVE AND NEGATIVE EFFECTORS OF EMBRYONIC AND POSTNATAL MUSCLE GROWTH IN THE CHICKEN AND THE MOUSE

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ABSTRACT

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Myostatin, belonging to the TGF-β family, negatively regulates skeletal muscle growth. The current study was conducted to assess the efficacy of *in ovo* administration of exogenous myostatin antagonist (MA) to enhance skeletal muscle growth and improve feed efficiency of broilers. Eggs were divided into three groups: uninjected control, vehicle-injected control and MA injected (600 ng per embryo). Eggs were injected once on either day 15 or 18 of embryogensis. *In ovo* administration of MA on day 15 resulted in the greatest increase in live weights (~8%, $P<0.10$), breast weights (~14%, $P<0.05$), leg weights (~10%, $P<0.10$) and shank length (~7%, $P<0.01$) in six-week old female broilers. Feed efficiency of the day 15 and day 18 MA injected mixed-sex broilers was unaffected at six weeks of age. Texture analysis showed the MA to have no effect on the shear force of the pectoralis muscle. Day 15 injected males did not have as pronounced effect as females in the measured variables. These findings suggest that *in ovo* administration of MA may provide a means to improve broiler productivity and profitability.

Growth hormone secretion is under the control of a pair of hypothalamic factors, growth hormone releasing hormone and somatostatin. The growth hormone secretagogue receptor (GHSR) and its endogenous ligand represent a novel third mechanism regulating the release of growth hormone. Early chicken embryonic development, prior to day 14, has been proposed to be independent of GH. However, recent evidence shows that peripheral GH secretion has paracrine/autocrine functions during embryonic development. In the current study, we used the reverse-transcriptase polymerase chain reaction to determine the expression pattern of the GHSR during embryonic development and the effects of *in ovo* recombinant human (rh) IGF-I administration on its expression pattern. Eggs were injected once with 100 ng rhIGF-I in 10 m*M* acetic acid, and 0.1 % BSA per embryo on embryonic day 3. Total RNA was isolated from whole embryos on embryonic day E0-6 (n=6 per day), thoracic/abdominal halves of the embryos on E7-E8 (n= 6 per day) and *Pectoralis* muscle on E9-E20 (n= 4 per day). We found that GHSR expression was low during E0-E4, followed by an increase on E5 and remained constant through E17. GHSR expression then increased on E18 before reducing on E20. A similar pattern was found in the rhIGF-I treated embryos with the exception of a significant increase in GHSR expression on E8. These data indicate that the GHSR may be active in regulating GH secretion during early embryonic development, and upregulation of the GHSR gene following IGF-I administration may have an important role in the determination of postnatal muscle growth.

Administration of the recently identified ligand of the growth hormone secretagoue receptor (GHSR) has been found to increase feed intake and fat deposition. During growth and aging, the myostatin knockout mouse has been shown to have a significant reduction in fat accumulation. In this study, we used RT-PCR to investigate the relationship between ghrelin and myostatin expression. Total RNA was isolated from

the brain, heart, pectoralis muscle, kidney and liver of nine-month-old myostatin knockout (n=3) and control (n=3) mice. Ghrelin expression was significantly reduced in the heart ($P<0.05$) and showed a trend for reduced expression in the pectoralis muscle ($P=0.07$) in myostatin knockout mice. However, ghrelin expression was not different in the brain, heart and liver between the control and myostatin knockout mice. These data indicate loss of myostatin may act by down regulating the expression of ghrelin to reduce the accumulation of fat in the myostatin knockout mouse. Additionally, these data are consistent with an autocrine/paracrine role for ghrelin, in the peripheral tissues, in metabolic regulation and nutrient partitioning.

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INTRODUCTION

From the beginning of embryology with Aristotle, the ability to physically study the chick embryo has proved a very powerful tool for many areas of embryonic development. Aristotle observed changes in the developing chicken by removing eggs from a hen's clutch during different times of incubation. The use of the chicken embryo has been indispensable in the establishment of the processes leading to muscle development. The first description of the primary unit embryo segmentation, the somite, was given by Marcello Malpighi in 1689, using a primitive microscope to observe changes in the chick embryos during early embryonic development. Although the African claw-toed frog (*Xenopus laevis*) has arguably made the greatest contributions to our understanding of embryonic development, the contribution of the chicken cannot be understated. In the mid 19th century, Robert Remark laid the framework for muscle development when he suggested the process of resegmentation, i.e. formation of several distinct regions, in the within the chick somite (reviewed by Brand-Saberi and Christ, 2000). Although, as I discuss further below, the chicken has been very important in the elucidation of vertebrate muscle development, a mistake in 1923 by a Dagsboro, Deleware hatchery added another reason to study chicken muscle development. In that year Cecile Steele, mistakenly received 500, rather than 50, replacement hens on her small farm in Delaware. When the birds became to large for the pens that she had, she was forced to slaughter the chickens and sell for meat for 62 cents per pound. This was the beginning of the now multi-billion-dollar broiler industry in America, and the impetus to study chicken muscle development for the chicken muscle.

LITERATURE REVIEW

Embryonic Origin of Muscle

The embryonic origin of body muscle cells in vertebrates can be traced back to the paraxial mesoderm in the primitive streak, which is formed during gasturalization of the early embryo. These cells undergo epithealization and segmentation to form the somites, the basic metameric unit of the embryo (reviewed by Keynes and Stern, 1988; Brand-Saberi and Christ, 2000). Somite segments form in pairs starting at the cranial end and moving cadually along the neural tube and notochord (reviewed by Christ and Ordahl, 1995; Brand-Saberi and Christ, 2000; Hirsinger et al., 2000). As the somite matures, the ventral region progresses through a transition from epithelial to mesenchymal cells making up the scleotome (Christ et al., 1979). The dorsal region maintains the epithelial identity forming the dermomyotome giving rise to both muscle cells and the deep layers of the skin.

Use of the Quail/Chick chimeras has allowed for the determination of myogenic progenitor cell sources in the somite (Ordahl and LeDourain, 1992; methodology review Ordahl and Christ, 1998). These studies have identified two distinct myogenic lineages originating from independent regions of the somite. The medial region of the dermomyotome contains the first cells to express markers of the myogenic fate, myoD, desmin and myosin (Williams and Ordahl, 1997). These cells form a layer of differentiated cells under the medial region of the dermomyotome, the myotome, which form the muscles of the back (Denetclaw et al., 1997). The muscles of the limbs and the abdominal cavity originate from cells in the lateral region of the dermomyotome (Ordahl

and LeDourian, 1992; reviewed by Christ and Ordahl, 1995; Brand-Saberi and Christ, 1999).

In addition to the location of cell origin, the quail/chick chimera experiments have found that cells in these regions of the early somite are not committed to this particular destination. That is, rotation (Aoyama and Asamoto, 1988), medial to lateral transplantation (Ordahl and LeDourain, 1992), or ventral to dorsal transplantation (Christ et al., 1992) of the early somites resulted in the development of the muscles according to the recipient location and not the donor location. These studies indicate that the myogenic cells of the somite interact with the surrounding tissues to determine the fate of the myogenic cells (reviewed by Brand-Sarbri and Christ, 2000)

Unlike the medial region of the demomyotome, which showed early expression of muscle specific proteins, the lateral region remained undifferentiated for almost two days beyond myotome differentiation and migrated out of the somite to form the limb and body wall muscles (Christ et al., 1977). Migration of the myogenic progenitor cells out of the lateral dermomyotome begins in the cranial edge of the somite, and eventually cells move out along the whole length (Jacobs et al., 1979; Jacob et al., 1978). At the limb buds, these myogenic progenitor cells move out of the somite in an undifferentiated form and become the ventral and dorsal premuscle masses in the limb buds (Schramm and Solursh, 1990).

Myogenic cell differentiation can be viewed as a process consisting of three steps (reviewed by Franzini-Armstrong and Fischman, 1994): 1. DNA synthesis ends (cells become post-mitotic), 2. cytoplasmic fusion, and 3. initiation the transcription of muscle specific proteins. This process leads to the development of large post-mitotic

multinucleated myotubes, with centrally located nuclei. As the expression of the muscle structural proteins increase, the nuclei take up a peripheral location in the cell, and show the striated appearance of mature muscle cells. Two distinct lineages of cells are used for growth of the large muscles, referred to as early (embryonic) and late (fetal) myoblasts forming the primary and secondary myofibers, respectively (Stockdale, 1990). The cell lineage can be differentiated by the size of and number of nuclei included in the myotubes formed *in vitro* (Seed and Hauschka, 1984), and by the specific myosin types expressed *in vivo* (Stockdale, 1990). In the chicken, primary myofibers are formed between days 3.5 and 6 (Stages 28 to 34, Hamburger and Hamilton, 1951) of chicken embryonic development (Fredette and Landmesser, 1991). In culture, these myoblasts differentiate into short myotubes containing a relatively low number of myonuceli (~30-40; Seed and Hauschka, 1984). In the limb bud, these smaller myotubes express both fast and slow type myosin heavy chain isoforms (Miller and Stockdale, 1986). The secondary myofibers, formed after day 7 (Stage 34), show the highest formation between embryonic day 7 and 17, when there is a 400-fold increase in the number of myofibers in the breast (O'Neil, 1987). The secondary myoblasts form much longer myotubes in culture containing upwards of 100 myonuceli (Seed and Hauschka, 1984). The primary myotubes are thought to create the scaffold over which the much larger secondary myotubes can grow and provide the bulk of the fibers in the mature muscle (reviewed by Hauschka, 1994). A summary of the key events in chicken embryonic development is given in figure I-1.

Investigation into the regulation of myogenic differentiation *in vivo* was hastened with the discovery of a family of basic Helix-Loop-Helix (bHLH) transcription factors

with the ability to transform fibroblasts *in vitro* into a myogenic lineage (Chio et al., 1990; Cserjesi and Olson, 1991; Weintraub et al., 1994). These Myogenic Regulatory Factors (MRFs) include four proteins MyoD, myf-5, myogenin and MRF-4, which are expressed at different times during avian muscle development (reviewed by Perry and Rudnicki, 2000). In the chicken, MyoD is expressed first followed very closely by myf-5, causing the cells to withdraw from the cell cycle and begin to express muscle specific proteins (Pownall and Emerson, 1992). MyoD and/or myf-5 activate the expression of myogenin and MRF-4, which have been found to function in maturation and maintenance of muscle cells, respectively (reviewed by Megeney and Rudnicki, 1995). Therefore, MyoD and myf-5 have been referred to as the myogenic determination factors, and myogenin and MRF-4 have become know as the maturation factors.

As mentioned above, the initial step in myogenic differentiation is withdrawal from the cell cycle prior to the expression of the muscle specific proteins and formation of the myotubes. Therefore, the process of proliferation and differentiation in the muscle cells are mutually exclusive processes during muscle growth. Interaction of the activities of proliferative and differentiative factor on the cells determines the number of mature myofibers in any given muscle. The interaction of growth factors on the maturation of the myoblasts has been show by the effects of serum concentrations in culture on the differentiation of myogenic cells. Depletion of the serum from the culture media results in the cells fusing to form the myotubes and expression of muscle specific proteins (Konigsberg, 1971). Using serum-free media with varing levels of specific growth factors has allowed the elucidation of theses growth factors effects on myoblast proliferation and differentiation (reviewed by Hauschka, 1994). Fibroblast Growth

Factors (FGF), Insulin-like Growth Factors (IGF) and Transforming Growth Factor-β (TGF-β) are perhaps the most extensively studied of these growth factors, and represent the variety of effects growth factors can have on myoblast proliferation and differentiation (reviewed by Florini and Margi, 1989; Ewton and Florini, 1990; Florini et al., 1991). FGF is a very potent stimulator of myoblast proliferation and inhibitor of differentiation. TGF-β is a very potent inhibitor of both myoblast proliferation and differentiation, and is thought to be the most potent inhibitor of proliferation. IGF-I, and to a lesser extent IGF-II, have been shown to stimulate both proliferation and differentiation of myoblasts in a biphasic dosage manner.

Although, tissue culture has proven helpful in the elucidation of the effects of growth factors on the myogenic differentiation process; however, the effective culture systems still required the influences of at least minor amounts of unidentified factors, typically supplied in serum supplements. The identification of additional factors expressed either within the developing muscles or secreted from surrounding tissues, such as the bone morphogenic proteins, Pax-3, and sonic hedgehog in the developing embryo, creates combinations of signals that as of now cannot be accurately replicated in culture (Amthor et al., 1999; reviewed by Buscher and Belmonte, 1999).

The discussion above indicates the complexity of the generation and maintenance of normal muscle growth. The interaction between positive and negative growth factors acts to determine both the number of muscle cells and the size of the fibers. The recently identified negative regulator of muscle growth, myostatin (also known as growth/differentiation factor-8) has been found to have a significant role in determination of the muscle size in mammals (Lee and McPherron, 1997; Bass et al., 1999). However,

the role of myostatin in regulation of muscle growth and development in the avian species has not received much attention. In contrast to the negative effects of myostatin, the somatotrophic axis (Hypothalamus→ Pituitary (growth hormone)→IGF) must be intact to stimulate muscle growth in the avian species. The regulation of GH secretion by the anterior pituitary in the avian species is regulated by the interaction of secreatory factors (growth hormone-releasing hormone and thyrotropin-releasing hormone) and inhibitors (somatostatin) (reviewed by Scanes 1997). An additional mechanism regulating the secretion of GH has been proposed with the identification of a group of peptides and non-peptidyl analogues known as the GH secretagogues acting through a common receptor (the growth hormone secretagogue receptor (GHSR)) (Reviewed by Smith et al., 1997). For this reason, the biological activities of myostatin (negative regulator) and the growth hormone secretagogues (positive regulators) have been reviewed in the following sections.

Myostatin

Beef cattle breeding has extensively selected for increased muscle mass. In Europe, the breeding regime has lead to the isolation of the 'double muscle' phenotype, which is a heritable trait characterized by a significant increase in the number of muscle fibers (Casas et al., 1998). An additional mammalian model of this phenotype was identified in the compact mouse (Varga et al., 1997). The genotype leading to this phenotype remained unresolved until the identification of a novel member of the transforming growth factor-β (TGF-β) super family, myostatin (McPherron et al., 1997). This group found that knocking out this gene resulted in a 200-300% increase in the

muscling of the mouse. Subsequent analyses have shown the double muscle and compact phenotypes are related to natural mutations in the myostatin gene. Since its identification 5 years ago, much has been learned about myostatin, however, there are still several unresolved questions that must be addressed to fully understand the role of myostatin in the regulation of muscle growth and development.

What is Myostatin?

Se-Jin Lee's group at John Hopkins University using degenerative PCR to identify novel members of the TGF-β family, identified a gene that they referred to as growth/differentiation factor-8 (GDF-8). Using homologous recombination, they generated a GDF-8 null mutant mouse, which showed a significant increase in muscle mass (McPherron et al., 1997). This increase in muscle mass was the result of both increased muscle cell number (hyperplasia) and increased muscle fiber size (hypertrophy). Therefore, GDF-8's role as a negative regulator of muscle growth led to its now common name, myostatin.

To date, more that 30 members of the TGF-β family have been identified. Members of the family have been found to have roles in the regulation of cell differentiation, proliferation and lineage determination (reviewed by Massague, 1998). Structurally, members of the TGF-β family share at least three common features (Figure I2; reviewed by McPherron and Lee, 1996). Myostatin is translated as a 376-aa protein showing all three of the hallmarks of other members of the TGF-β family. First, the N-terminus contains a number of hydrophobic amino acids, which function as a signal sequence for secretion. Myostatin's first 40 amino acids show a high proportion of

hydrophobic residues, indicating the presence of this element. Second, the presence of a conserved RSRR proteolytic recognition site in the C-terminal domain indicates the secretion of the protein in an inactive form, which must subsequently undergo proteolytic cleavage for activation. This sequence is present between amino acids 264-267 in myostatin. Additionally, western blot analysis of purified myostatin proteins under reducing conditions shows small (~12.5 kDa) and large (~36 kDa) bands, reflecting the presence of the propeptide and the activated C-terminal monomer (Lee and McPherron, 2001). The final general characteristic of the TGF-β proteins is the presence of six to nine cysteine residues in the C-terminus, which are responsible for 'cysteine knot' formation during homodimerization; myostatin contains nine cysteines in its C-terminus. Western analysis of purified myostatin verified the homodimerization with the ~12.5 kDa monomers detected under the reducing conditions and ~25 kDa under the non-reducing conditions (Lee and McPherron, 2001).

TGF-β family members signal through cell surface receptor activation to elicit their biological responses (Figure I3; reviewed by Massague, 1998). Binding of a homodimer of the activated C-terminal to the TGF-β type II receptor recruits the type I receptor to modulate gene expression of the cells. Using radioiodinated myostatin, Lee and McPherron (2001) found that myostatin also binds to the activin receptor type II. More specifically, they found that the affinity of myostatin was highest for the activin receptor type-IIB (ActRIIB). This finding provides further evidence that myostatin is a member of the TFG-β family (Lee and McPherron, 2001). Additionally, expression of a dominant negative ActRIIB in the mouse causes phenotypic changes in the mouse similar to the myostatin knockout mouse, indicating active signaling though this receptor.

Where is Myostatin Expressed?

As mentioned above, the myostatin null mouse shows a significant increase in muscle mass (McPherron et al., 1997) indicating a major role in the regulation of muscle cell proliferation. The 100% conservation of the sequence within the biologically active C-terminal domain of the human, murine, rat, porcine, chicken, and turkey indicates a remarkably conservation of function amoung these species (McPherron et al., 1997). Embryonic expression pattern for myostatin in the mouse (McPherron et al., 1997), cattle (Kambadur et al., 1997) and chicken (Kocamis et al., 1999b) all reflect the muscle specific role for myostatin during embryonic development. In the mouse, the initial expression of myostatin was detected in the myotome region of the somite, moving out into the limb and body cavity as the myoblasts began to mature (McPherron et al., 1997). In cattle, Kambadur et al., (1997) found low myostatin expression before day 29 of gestation, which increased on day 31 and remained high in the developing muscle. This time period is consistent with the timing of primary myoblast differentiation in the bovine. Additionally, the dramatic increase in the expression of myostatin on embryonic days 7 and 17 during chicken development overlaps with the approximate timing of primary and secondary muscle fiber formation (Figure I1; Kocamis et al., 1999b). They also observed a reduction in myostatin expression just prior to hatch, when muscle development is most likely completed.

Post-natally, myostatin expression appears primarily in skeletal muscle (McPherron et al., 1997; Kambadur et al., 1997). In murine samples, significantly higher expression has been found in the *Planteris* and the *Gastrocnemius* as compared to the

Soleus (Wehling et al., 2000; Carlson et al., 1999), indicating higher myostatin expression is associated with fast type muscle fibers. Similarly, in cultured myoblasts, the expression of myostatin was associated with the myotubes expressing the fast type myosin heavy chain (Artaza et al., 2002). To further expand the role of myostatin in muscle growth and development, up regulation of myostatin was reported in conditions leading to muscle wasting including, limb unloading (Wehling et al., 2000; Carlson et al., 1999), exposure to zero gravity during space flight (Lalani et al., 2000), extended periods of bed rest (Reardon et al., 2001), aging (Marcell et al., 2001), and advanced HIV (Cadavid-Gonzales et al., 1998).

Outside the skeletal muscle, lower levels of myostatin expression has been reported in adipose tissue (McPherron et al., 1997), Purkinje fibers of the heart (Sharma et al., 1999) and in the tubuloalveolar secreatory lobules of the lactating mammary gland of pigs (Ji et al., 1998). The significances of this requires further investigation.

How Does Myostatin Function?

The limited expression of myostatin outside the skeletal muscle indicates myostatin may be acting in an autocrine/paracrine manner in the regulation of skeletal muscle proliferation (McPherron et al., 1997). Several studies have shown myostatin to reduce myoblast proliferation in culture (Thomas et al., 2000; Taylor et al., 2001; Rios et al., 2001; Artaza et al., 2002) consistent with the loss of active myostatin leading to the 'double muscle' phenotype. Actively dividing cells are dependent on the activity of the cyclins and cyclin-dependant kinases (CDK) for progression through the cell cycle. Thomas et al. (2000) found upregulation in the transcription of the CDK inhibitor (CDI)

p21 in C2C12 cells treated with exogenous myostatin. Therefore, attenuation of the CDK activity clearly leads to withdrawal of the myoblasts from the cell cycle leading to the anti-proliferative effects of myostatin. Recently, Zimmers et al., (2002) found *in vivo* over-expression of myostatin, by the induction of myostatin expressing tumors, that increased myostatin lead to a cachxia-like phenotype in the mouse. This demonstrates that myostatin is active in reducing the proliferation of myoblasts *in vivo*. This also indicates a systemic effect of myostatin, which is addressed below.

Unlike the clearly defined role of myostatin in myoblast proliferation, the effect of myostatin on cell differentiation and survival (apoptosis) has not been clearly defined. Initially, there was shown to be no visible change in myoblast differentiation *in vitro* with myostatin treatment (Thomas et al., 2000). However, the effects on muscle differentiation were shown with the reduced protein synthesis in C2C12 cells treated with myostatin (Talyor et al., 2001). Additionally, Rios et al., (2002) found that myostatin decreased expression of markers of muscle cell differentiation, myogenin and creatine kinase. Similar to the confounding results on myoblast differentiation, the effect of myostatin on cell survival has not been consistent. Using the TUNEL assay and cell sorting, Thomas et al. (2000) were unable to show any apoptotic or anti-apoptotic effects of myostatin. A more recent study showed myostatin was able to reduce to cell death in the same type of cell culture (Rio et al., 2001). These confounding result are intriguing and warrant further investigation. Collectively, the data above indicate myostatin acts to reduce myoblasts proliferation in culture and provide evidence that *in vivo* loss of active myostatin leads to increased muscle.

Generation of a myostatin knockout mouse (McPherron et al., 1997) and identification of natural mutations in the myostatin gene in mice (Szabo et al., 1998) and cattle (McPherron and Lee, 1997; Smith et al., 1997; Grobet et al., 1998) show that myostatin attenuation leads to a 'double muscle' phenotype. Therefore, methods to attenuate myostatin activity *in vivo* may prove useful in the treatment of muscular diseases. The activity of TGF-β family members is regulated by proteolytic cleavage, dimerization and interaction with the receptors (see figure I2). Analysis of the mutation in the Piedmontese and Belgian Blue cattle indicate that a reduction in myostatin activity presumably leads to the double muscle phenotype. In the Piedmontese, a 2-bp mutation in the gene results in the substitution of tyrosine for the cysteine at residue 313. This presumably acts to prevent 'cysteine knot' formation during dimerization, therefore, attenuating the activity of myostatin (McPherron and Lee, 1997; Kambadur et al., 1997). Similarly, an 11-bp mutation in the Belgian Blue myostatin gene results in a truncated myostatin protein. Similarly, the generation of a mouse with an altered proteolytic cleavage site on myostatin showed a significant increase in muscle mass (Zhu et al., 2000). Therefore, attenuation of myostatin activity results in an increase in the muscle mass regardless of the method by which endogenous myostatin activity is blocked.

The binding of the active protein to the cleaved prodomain (also known as latency-associated protein) or binding proteins (e.g. follistatin, noggin and α-2-macroglobulin) may also attenuate TGF-β family member activity (Figure I2; reviewed by Piek et al., 1999). In receptor binding assays, both follistatin and the N-terminal propeptide were found to significantly reduce myostatin receptor binding (Lee and McPherron, 2001). Creation of transgenic mice over expressing either follistatin (Lee

and McPherron, 2001) or the prodomain (Yang et al., 2001) under the direction of a muscle specific promoter, lead to a significant increase in muscle mass. In summary, attenuation of myostatin activity may be achieved through manipulation of the regulatory mechanisms controlling activity of myostatin protein. Understanding of these mechanisms may have important implications in human health and agricultural production.

Non-muscle Roles of Myostatin?

Generation of the myostatin knockout mouse and identification of the mutation leading to the 'double muscle' phenotype have led to investigations of myostatin that specifically focus on the paracrine/autocrine effects of myostatin on the growth and development of muscle (Lee and McPherron, 1999; Bass et al., 1999). However, more recent data indicate that myostatin may have roles reaching far beyond muscle alone and may be an actively circulating growth factor. In fish, two different myostatin proteins have been isolated (Roberts and Geitz, 2001; Rodgers et al., 2001; Rescan et al., 2001) with expression reported in a variety of tissues outside of muscle. One of the isoforms was found to be ubiquitously expressed in fish tissues, the other was limited to expression in the skeletal muscle and brain (Rescan et al., 2001; Ostbyte et al., 2001). This suggests, at least in the lower vertebrates, a more extensive role for myostatin in growth, development or other processes.

Nutrient partitioning isof particular interest to the agricultura industry to improve the value of the product produced. As mentioned earlier, a low level of myostatin expression was reported in fat tissues of mice (McPherron et al., 1997). However, until

recently, this expression was considered insignificant. Treatment of 3T3-L1 preadipocytes with myostatin reduced expression of CCAAT/enhancer binding protein alpha (C/EBP alpha) and peroxisome proliferator-activated receptor gamma (PPAR gamma), the main proteins involved in adipocyte differentiation (Kim et al., 2001). Similarly, Lin et al., (2002) found that the regulation of these same genes was altered in the myostatin null mouse. They concluded that these changes were the cause of the reduced adipogenesis observed during growth of the mouse. Crossing of the myostatin knockout mice with either the Agouti lethal (A^y) or the Obese (*ob/ob*) mice, animal models of obesity and diabetes, not only resulted in a significant reduction in fat deposition in the hybrids, but also showed changes in the animal's glucose tolerance (McPherron and Lee, 2002). We have recently found IGF-II gene expression was significantly increased in the kidney and *soleus* muscle of the myostatin knockout mouse (Kocamis et al., 2002). This may indicate one mechanism by which metabolism is altered. That is, IGF-II is able to bind to and activate the insulin receptor, therefore IGF-II acting through the insulin receptor may cause, at least in part, the observed changes in the fat deposition and glucose metabolism (Lin et al., 2002; McPherron and Lee, 2002). Thus, alterations in fat deposition and metabolism in the mouse resulting form myostatin attenuation may be the interaction of direct and indirect activities on the fat tissues.

A systemic role of myostatin has been the source of some debate, in part because the ability to detect myostatin in the circulation has been hindered by the lack of available antibodies available. In 1998, Cadavid-Gonzales et al., were the first to indicate the presence of myostatin proteins in the circulation of humans, which were increased during HIV induced muscle wasting. However, the protein they identified as myostatin was

~30kDa, much larger than the ~12.5 kDa myostatin monomer and the ~25kDa dimer (Lee and McPherron, 2001). Using a newly developed antibody specific for the C-terminus of myostatin, Zimmers et al., (2002) were able to show a measurable amount of the specific myostatin monomer, at ~12.5 kDa, in the wild-type mouse, a protein not identifiable in myostatin knockout mouse serum. In the same study, establishment of CHO-myostatin tumors in the muscles resulted in a cachexia–like response, which was not limited to the muscle in which the tumors grew further supporting a systemic role for myostatin. Therefore, myostatin blocking agents, receptor agonists or myostatin binding proteins, may be effective treatments for muscle wasting conditions in humans.

The presence of the Belgian Blue 11-bp mutation in the South Devon cattle population, which is not considered as double muscled, indicates that interaction with other genetic factors (i.e. genetic background) are necessary (Smith et al., 2000). Additionally, both the Callipyge sheep (Freking et al., 1998) and the Pietrain Pig (Brenig and Brem, 1992) show increased muscling, but the markers of this type of muscle hypertrophy have been found in regions of the genome independent of the myostatin location. This provides evidence that the genetic background of the animal that carries a mutation in myostatin determines the degree of muscular growth. Some factors that may be involved in this include genes regulating the proliferation of the muscle cells, i.e. GH, IGF's and/or FGF's or those proteins that interact directly with myostatin or the components of its signaling pathway (see Figure I3).

Growth Hormone/IGF-I and Growth

Somatic growth, defined as the increase in body size or weight, is regulated by the interaction of external (environmental and nutritional) and endogenous (hormonal) stimuli. A fundamental role for growth hormone (GH; also known as somatotropin) is well established as a hormonal regulator of postnatal growth. The somatotroph cells of the anterior pituitary serve as the site of GH production and secretion. In most vertebrate species, hypophysectomy results in a significant reduction in body weight gain, bone growth and reduced growth of several organs (reviewed by Scanes et al., 1986), which can be mostly overcome by supplementation with exogenous GH (reviewed by Butler and LeRoith, 2001). Similarly, many of the genetic causes of growth retardation in animals, both mutations and knockouts, are found within the GH system (reviewed by Sellier, 2000). For example, Laron-Type Dwarfism and the sex linked dwarf (SLD) chicken have severe growth retardation resulting from a mutation in the GH-receptor gene (GH-R; reviewed by Hull and Harvey, 1999).

GH, belonging to the large family of cytokine peptides, has been proposed to have evolved from a common ancestral gene with prolactin and placental lactogen (reviewed by Miller and Eberhardt, 1983). In the chicken, GH is translated as a 216-aa peptide, containing a 25-aa signal sequence on the N-terminus, and a 191-aa mature protein, which shows 79% homology to the rat GH sequence (Lamb et al., 1988). The mature protein has a molecular weight of approximately 22 kDa and contains two disulfide linkages, which are proposed to have an important role in determination of its secondary structure and therefore ligand-receptor interactions (reviewed by Kopchick and Chen, 1999). The effects of GH on the cell are mediated through the transmembrane growth

hormone receptor (GH-R) by activating the JAK/STAT signal transduction pathway (reviewed by Carter-Su et al., 1996).

Early studies proposed that GH's effects on growth were mediated through an intermediate or a somatomedin to promote growth. This theory had become commonly referred to as the 'somatomedin hypothesis' (Salmon and Daughaday, 1957; reviewed by LeRoith et al, 2001). The purification of the insulin-like growth factor-I (IGF-I), named for its ability to stimulate glucose uptake by fat and muscle cells, provided the intermediate for the mediation of GH effects to the target tissues (Rinderknecht and Humbel, 1978; Klapper et al., 1983). Not only was IGF-I found to be a potent mitogen, but its secretion from the liver is regulated by GH. Therefore, the somatomedin hypothesis stated that GH secretion from the anterior pituitary regulated the secretion of IGF-I by the liver, mediating the effects to the target tissues (Figure I4; reviewed by LeRoith et al., 2001). The original somatomedin hypothesis was challenged in the 1980's by the discovery of IGF-I expression in extrahepatic tissues, which was regulated by GH (D'Ercole et al., 1984; Roberts et al., 1987). In 1985, Green et al., reported the effects of GH and IGF-I on 3T3 pre-adipocytes were exclusive. That is, GH stimulated differentiation of the pre-adipocytes, whereas IGF-I resulted in the expansion of the cell populations, a finding that was supported by the effects reported on longitudinal bone growth and chondrocyte differentiation (reviewed by Isaksson et al., 1987). From this, a 'dual effector' theory was proposed to describe the activity of the GH/IGF-I axis on growth in the animal, which proposed that GH acted directly on the cells to determine the cellular fate and induced the expression of IGF-I that allowed for the clonal expansion of the cells. The discovery of expansion of chondrocytes in the IGF-I knockout mouse

indicates that the effects of GH on longitudinal plate growth are independent of GH (Wang et al., 1999). Even though this likely disproves the true 'dual effector' theory, the discovery that GH is able to induce IGF-I expression in extrahepatic tissues is very important in understanding growth regulatory mechanisms of GH *in vivo*. The evaluation of the GH/IGF-I axis described above is illustrated in Figure I4.

In most farm animals, exogenous GH has been found to significantly increase growth rate, improve feed conversion efficiency, increase lean tissue accretion and decrease fat deposition (reviewed by Etherton and Bouman, 1998). This is likely a reflection of the metabolic effects of GH, which is most evident on adipose and muscle cell metabolism (reviewed by Florini et al., 1996; Butler and LeRoith, 2001). The reduced adipocyte volume (Lee et al., 1994) and reduced lipid content of the muscle (Lafaucheur et al., 1992) indicate that chronic GH administration reduces lipid deposition. In cattle (Peters, 1986) and swine (Wolverton et al., 1992), this response was proposed to be the result of reduced lipogensis. However, in the chicken, the same effect appears to be the result of increased lipolysis in the adipose tissues (Vasilatos-Younken et al., 1988). Additionally, chronic GH administration reduces blood urea nitrogen in cattle (Eisemann et al., 1986) and pigs (Etherton et al., 1987) indicating a reduction in the total breakdown of amino acids. GH treatment has been found to increase protein accretion in cattle hind limb muscle by as much as 53% (Boisclair et al., 1994). Whether these effects are direct or mediated thought the IGF's is a matter of some controversy. In the muscle it appears the increased growth and protein accretion can be largely attributed to the action of IGF-I, produced both locally and systemically (reviewed by Florini et al., 1996). However, the expression of the GH-R in the perpherial tissues, allows for some direct

action on the cells, even if it is just to increase cellular responsiveness to lipolytic signals (reviewed by Scanes, 1999).

Unlike the result in large farm animals indicated above, the results of GH administration to the chicken have not been consistently observed (reviewed by Scanes et al., 1986; Vasilatos-Youken et al., 1999). This may be the result of the relationship between GH and thyroid hormone levels in the chicken. The activity of thyroid hormone in the chicken results from the interaction of activation and inactivation of thyroxine (T4), the primary form of thyroid hormone secreted by the thyroid gland. The metabolically active form 3,3',5,-triiodothyronine (T3), is formed by outer ring deiodination catalyzed by iodothyronine deiodinase type I and type II primarily in the liver. The inactivation of T4 to rT3 (or T3 to T2) by the inner ring deiodination is catalyzed by type I and type III deiodinases (reviewed by Darras et al., 2000). The SLD chicken is characterized by high GH and low IGF-I serum concentrations, show a significant reduction in circulating concentrations of GH following T4 treatment (Tixier-Boichard et al., 1990). Also, in a hypothyroid state, the elevated GH concentrations observed were not matched by increases in the circulating levels of IGF-I (Decuypere et al., 1987). Additionally, the same study found administration of T3 resulted in a reduction in GH concentration without further lowering the IGF-I concentration. These data suggests that the concentration of the thyroid hormones may alter the liver sensitivity to GH and thereby alter the secretion of IGF-I (reviewed by Vasilatos-Younken et al., 1999). Therefore the interaction between GH and the thyroid hormones has been proposed to have a major effect on the growth of poultry.

In addition to the effects of thyroid hormone on the GH related effects on growth, GH may also be acting to regulate the levels of thyroid hormone, having significant consequences on the metabolic regulation of the birds. A significant effect of GH on the levles of the individual forms of the thyroid hormones has been demonstrated using both exogenously supplied GH (Vasilatos-Younken et al. 2000) and in a natural model (the naked neck chicken, Gonzales et al., 1999). The pulsatile infusion of cGH (100 μg/kg body weight) resulted in a significant increase in the serum T3 without altering T4 (Vasilatos-Younken et al., 2000). They found a significant reduction in the hepatic Type III deiododinase activity in response to the GH infuson, which explains the increase in circulating T3 concentrations in the birds. Additionally, the naked neck chicken, characterized as having lower feed conversion efficiency and much slower growth rate than the commercially available broiler strains (Gonzales et al., 1998), was shown to have higher GH concentration during the rapidly growing period (hatch through week 7; Gonzales et al., 1999). These chickens had higher serum T3 and T3/T4 ratio during this same period. The induction of a hyperthyroid (high T3/T4 ratio) state resulted in a decline in the cirulationg IGF-I concentration (Goddard et al., 1988). Taken together, these data demonstrate that the hyperthyroid state of chickens with elevated GH may attenuate the effects of GH on whole animal and muscle growth.

The comparatively late development of the anterior pituitary suggests that much of embryonic development occurs independent of growth hormone. In the chicken, the exact timing of somatotroph differentiation is a source of some debate. The first detectible GH secreting cells in the anterior pituitary have been found at two different times E4.5 (Thommes et al., 1987) or E12 (Jozsa et al., 1979, Malamed et al., 1993).

However, using the reverse hemolytic plaque assay, Porter et al. (1995) determined that GH secretion in somatotroph cells was limited before E12. Similarly, the ability of GH releasing hormone to induce GH secretion during embryonic development was significantly limited before E14 (Darras et al., 1994). The lack of detectable serum growth hormone until embryonic day 17 (E17) (Harvey et al., 1979) and pituitary GH mRNA until E18 (McCann-Levorse et al., 1992) has lead to the conclusion that early embryonic chicken development is GH-independent.

However, Harvey et al. (2000) found GH-immunoreactivity and GH Receptor (GHR)-immunoreactivity throughout the peripheral tissues of E3, E6 and E7 chick embryos. Additionally, mRNA expression of GH from whole embryo was detected during this time period, well before differentiation of the somatotroph (E16, Porter et al., 1995), suggesting an anterior pituitary-independent role for GH during early embryonic development (Harvey et al., 2000, Murphy and Harvey, 2001). Expression of the GH regulated gene-1 (GHRG-1) in many, but not all, tissues on E8 shows active signalling through the GH-R during embryonic development, supporting an autocrine/paracrine pattern of signalling during early embryonic development (Harvey et al., 2001). Therefore, modulation of GH synthesis during early embryonic development may provide a method to alter postnatal growth.

The pulsatile release of GH is regulated by the complex interaction of stimulatory and inhibitory factors acting on the somatotroph cells of the anterior pituitary (Figure I5; reviewed by Muller et al, 1999). In all vertebrate species studies, the primary control of GH secretion is regulated by a pair of hypothalamic factors, the GH-releasing hormone (GHRH; stimulatory) and somatostatin (SS; inhibitory). In the chicken, in addition to

GHRH, an additional hypothalamic stimulatory factor has been identified thyrotropin-releasing hormone (TRH; reviewed by Scanes et al., 1984). In addition to these local signals, both GH and IGF-I have been found to inhibit the secretion of GH through inhibition of GHRH in the hypothalamus and directly on the somatotroph cells of the anterior pituitary (reviewed by Muller et al., 1999). In the chicken, as opposed to mammals, thyroid hormones, particularly T3, have been shown to be a potent inhibitors of GH secretion (reviewed by Scanes et al., 1984). This effect is particularly evident in the SLD chicken, in which the high concentrations of GH in the animal are returned to approximately the same level as the normal chicken with exogenous T3 in the feed (Harvey, 1983). The complex regulation of the secretion of GH represents the interaction between a variety of factors some which have yet to be identified.

What are the Growth Hormone Secretagogues (GHS)?

In the late 1970's a group at Tulane University lead by Cyril Bowers were investigating the opiate potential of Met^5-enkephalin derivatives and found them to have very low or no opiate potential. However, they found that these peptides had the ability to induce growth hormone secretion from the pituitary (Bowers et al., 1980). Therefore, they were referred to as the GH releasing peptides (GHRP). Starting with Met^5-enkephalin (Tyr-Gly-Gly-Phe-Met-NH_2) and Leu^5-enkephalin (Tyr-Gly-Gly-Phe-Leu-NH_2), this group investigated the secretory potential of a wide variety of sequence and structurally related synthetic peptides (Momany et al., 1981; 1984). Several of these analogues were shown to induce GH secretion in perfused pituitary cell culture, but were not able to increase serum GH concentrations *in vivo*. The use of conformation energy

calculations and nuclear magnetic resonance allowed the identification of the structural components of the peptide and significantly advanced the rate of discovery of more potent peptides (Momany et al., 1984). These methodologies lead to the identification of a hexapeptide (His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2; also known as GHRP-6) with the ability to specifically cause GH release both *in vitro* and *in vivo* (Bowers et al., 1984). Over the next 15 years, a wide variety of peptides were identified with increasing potencies to increase circulating growth hormone (Figure I6; reviewed by Smith et al., 1997). In addition to GHRP-6, three additional peptides GHRP-1 (Ala-His-D-β-Nal-Ala-Trp-D-Phe-Lys-NH_2), GHRP-2 (D-Ala-D-β-Nal-Ala-Trp-D-Phe-Lys-NH_2), and Hexarelin (His-D-2-MeTrp-Ala-Trp-Phe-Lys-NH_2) induce GH secretion in a very potent manner (reviewed by Smith et al., 1997; Bowers et al., 1999).

Although the GHRPs functioned very well to induce the secretion of GH, their functionality was significantly limited because of low oral bioavailability. Therefore, two drug companies, Merck and Genentech, used the structural information available to formulate compounds that could mimic GHRP. Collectively, these peptides and analogue have become referred as the Growth Hormone Secretagogues (GHS). Three classes of these non-peptidyl GHSs have been identified based on the internal structural bases (Figure I7). The benzolactams (Merck drug L-692,429) and the spiroindanes (Merck drug MK-0677) were based on the central structure of GHRP-6 (reviewed by Smith et al., 1997). On the other hand, Genentech used the core of GHRP-2 to design the isonipecotic GHSs (Genentech drugs G-7302 and G-7039; Elias et al., 1995). Each compound functions through a mechanism distinct from that of the GHRH and therefore is thought to act through a GHS receptor (see below). The discovery of these non-

peptidyl forms of the GHS allowed for an orally available form of the GHS, but had not really increased the understanding of the mechanism by which GH secretion was induced.

How do the GHS Function?

The GH secratory activity of the GHS had been identified before the identification of the endogenous GHRH, and for this reason the action of the GHS was believed to act through the yet to be identified GHRH mechanism (Smith et al., 1980). However, the almost simultaneous identification of a 40-aa (Rivier et al., 1982) and a 44-aa (Guillemin et al., 1982) proteins secreted from pancreatic tumors of patients with acromegaly lead to the identification of the endogenous GHRH (known at the time as GH releasing factor) in the hypothalamus. However, treatment of pituitary cells with the GHRH agonist did not attenuate the ability of GHRP-6 (Cheng et al., 1989), Hexarelin (Bowers et al., 1991) or MK-0677 (Patchett et al., 1995) to induce GH secretion. Additionally, cells treated with GHRH alone, resulted in an increase in intracellular cAMP levels, a result that was not reflected in the cellular treatment with either GHRP-6 (Cheng et al., 1989) or MK-0677 (Patchett et al., 1995). These data suggested that the GHS and GHRH were acting via different mechanisms to induce the release of GH. Alternatively, the GHS may act in the pituitary or hypothalamus to reduce the sensitivity to or secretion of SS. This possibility was eliminated by the demonstration that rats passively immunized against SS show elevated serum GH concentrations with the administration of GHRP-6 or Hexarelin (Conley et al., 1995). In the same study, they had shown the GHS acted to increase

intracellular Ca^{++} concentration, a result not mimiced by treatment with GHRH. Thus, the activity of the GHS was suggested to act via their own receptor (Figure I8).

Using radiolabeled [^{35}S]MK-0677, Pong et al. (1996) identified a specific fraction of porcine and rat pituitary membranes with that bound this GHS. Additionally, they found this binding was not displaced with addition of excess unlabeled GHRH, but was displaced by GHRP-6 and Hexarelin. Therefore, the existence of a specific GHS receptor (GHSR) with overlapping affinity was proposed. This receptor was identified by Howard et al., (1996) using a reverse pharmacological technique. They injected poly-A RNA from porcine pituitaries into *Xenopus oocytes* and monitored changes in the intracellular Ca^{++} concentrations following MK-0677 treatment to identify cells containing the mRNA encoding the GHSR. From this, they were able to identify the porcine GHSR, which encoded a 366 aa protein, containing sequence consistent with the G-protein coupled seven transmembrane domain (7-TMD) receptor. In addition to the full-length receptor, termed GHSR 1a, a truncated mRNA was identified encoding a 253-aa protein containing the first 5-TMDs. Similar to the membrane-binding assay of Pong et al., (1996), this study found an inability of GHRH to displace labeled MK-0677 from COS-7 cells expressing this receptor. Additonally, both GHRP-2 and GHRP-6 were shown to displace MK-0677 from these cells, indicating this is also the same receptor utilized by the peptide GHS. Based on the homology, this protein was classified into the neurotensin family of receptors (53-58% homology). The ligand binding domain of the GHSR is highly conserved, showing a 58% homology between the human and the puffer fish (*Spheroides nephelus*), which are separated by more than 400 million years evolutionarily (Palyha et al., 2000). They also suggested a functional conservation by the binding of the

GHS to the receptor in both species. Subsequently, three other receptors (GPR38, GPR39 and FM-3) have been identified showing homology only to the GHSR (McKee et al., 1997), indicating the potential for more than one receptor with the ability to bind the GHS.

Identification of the specific GHSR allowed for clarification of the differences between the GHSR and the GHRH receptor (GHRHR) in the pituitary (Figure I8). That is, each of these GH releasing stimulators were found to signal through 7-TMD receptors using a Trimeric G-Protein mechamnism, however their methods of signal transduction were different. The GHRH utilized the α_s subunit of the G-protein to activate adenylyl cyclase and cAMP synthesis as the 2nd messenger system to stimulate GH secretion. Alternatively, the GHSR utilizes the α_{11} and the $\beta\gamma$ subunits to activate phospholipase-C$_\beta$ and IP_3 to increase intracellular Ca^{++} concentrations resulting in the secretion of GH (reviewed by Smith et al., 1997). The existence of this orphan receptor with a unique signal transduction pathway lead to the proposal of an endogenous ligand that acts in an independent manner to regulate GH secretion and the identification of the related receptor lead to the possibility of other roles.

Expression data for the GHSR has been inconsistent, which may be attributed to the low expression of the receptor even in presumptive target tissues (pituitary and hypothalamus; reviewed by Howard et al., 2000). However, there is clear evidence showing the expression of this receptor in the pituitary and several hypothalamic nuclei, including nuclei not involved in the regulation of GH secretion (Guan et al., 1997). In this same study using the ribonuclease protection assay, they were unable to identify expression in peripheral tissues, with the exception of a weak, but consistent signal from

the pancreas. Using real-time PCR, Gnanapavan et al., (2002) found human GHSR 1a expression primarily in the pituitary, with lower expression in the thyroid, pancreas, spleen, myocardium and adrenal gland. However, they found the expression of the presumed non-functional GHSR 1b in most of the tissues sampled. Papotti et al. (2000) found Hexarelin binding to receptors in a variety of tissues, with the highest binding found in the heart and not the brain. Taken together, these data suggest that the active GHSR may not be the only protein actively binding the GHS, and the subsequent identification of the other receptors may elucidate some of the non-GH related activities of the GHS (see below).

Investigations into the effects of the GHS in chicken growth and development have been significantly limited, however, there is data to support the existence of a GHSR system in avians. Bowers et al., (1984) showed that GHRP-6 was effective in stimulating GH secretion in the chicken following pretreatment with excess SS. However, this response was much lower than similarly treated mammals. A more recent study, using the more potent non-peptidyl GHS (Merck's L-692,429), found increased GH secretion both directly at the anterior pituitary and through stimulation of TRH secretion from the hypothalamus (Geris et al., 2001). Additionally, Toogood et al. (1999) identified a cDNA sequence with significant homology to the human GHSR from a chicken pituitary cDNA library. Together, these data indicate the presence of the GHSR in the pituitary of the chicken acting to increase the quantity of GH released.

What is the Endogenous GHS?

Two groups almost simultaneously reported the same gene product as being different proteins with completely different suggested roles. Tomasetto et al., (2000) in searching for new gastric proteins to study the function and differentiation of the stomach, stumbled upon the endogenous ligand of the GHSR. Using cDNA library screening techniques, this group identified a cDNA encoding a 117-aa propeptide that showed significant homology to motilin, a regulator of gastric motility. This novel protein, designated motilin-related peptide, was found to be primarily expressed in the endocrine cells of the stomach. They proposed a role for this peptide in the regulation of gastric motility and secretion.

Meanwhile, a Japanese group set out to identify the endougenous ligand for the GHSR (Kojima et al., 1999; reviewed by Kojima et al., 2001; Pombo et al., 2001). Using a CHO cell line transfected to express an active GHSR, they screened the ability of protein extracts from a variety of tissues to activate the GHSR by monitoring changes in the intracellular Ca^{++} concentration. Much to their surprise, brain extracts, even when applied to the cells in high concentrations, were unable to significantly alter intracellular Ca^{++} conentrations, but only 1 mg of stomach proteins was required to elicit a significant response. Using RP-HPLC, the specific protein binding to the GHSR was purified. The sequence of this 28-aa peptide contained an unidentifiable residue in position 3. Complementary DNA analysis suggested this amino acid was a serine residue, however, the construction of a synthetic peptide with a serine residue at position 3 was unable to bind to the receptor. Additionally, this peptide was 126 mass units smaller than the purified form of the peptide. After attempting a variety of modifications, they found that

the addition of an octanoylate residue, an 8-C fatty acid, residue allowed for proper binding to the receptor and the correct MW of the protein. This protein was called ghrelin, from 'ghre' the Proto-Indo-European root of the word 'grow'. Nucleic and amino acid analysis showed that the motilin-related peptide of Tomasetto et al., (2000) and preproghrelin of Kojima et al., (1999) were exactly the same protein (Figure I9 commented on by Del Rincon et al., 2001). The protein is translated as a 117-aa prepropeptide and presumably transported to the golgi apparatus for proteolytic modification and addition of the octanoylate residue to the ser-3 to form the active 28-aa peptide for secretion. Ghrelin represents the first protein identified to contain a medium chain fatty acid modification (Kojima et al., 1999). This is speculated to allow peripheral ghrelin to cross the blood/brain barrier in order to access the pituitary and hypothalamus. Additonally, this is thought to provide receptor specificity and allow for receptor binding (discussed further below).

Subsequent analysis of protein samples from the stomach led to the identification of an alternative form of ghrelin, differing from ghrelin only in the exclusion of the glutamine residue at amino acid 14 and therefore termed des-Gln^{14} ghrelin (Hosoda et al., 2000a). Using Southern Blot and genomic sequence analysis, they determined that this was the result of splice variation and not a closely related gene. Another ghrelin gene derived transcript (GGDT) has been identified in the testis of the mouse (Tanaka et al., 2001). This 320-bp transcript contains a unique 68-bp 5' sequence and a 252-bp 3' sequence identical to the ghrelin cDNA. The protein translated from this transcript and the function of this peptide has yet to be elucidated.

Using Northern Blot and *in situ* hybridization, Kojima et al., (1999) found ghrelin expression was limited to the endocrine cells of the stomach and the arcuate nuclei of the hypothalamus. However, subsequent studies using much more sensitive reverse transcription PCR were able to identify ghrelin expression in a variety of peripheral tissues, including pituitary, kidney, heart, skeletal muscle, liver, spleen and adrenals (Date et al., 2000; Mori et al., 2000; Gnanapavan et al., 2002). In all of these studies the highest expression of ghrelin was found in the stomach, and the upper gastrointestinal tract organs. Additionally, ghrelin expression in the placental tissues has led to the proposal of a role for ghrelin during embryonic development (Gualillo et al., 2001).

What are the In Vivo Functions of Ghrelin and the GHSs?

I*n vitro* studies (Kojima et al., 1999, Hosoda et al., 2000) and *in vivo* in rats (Seoane et al., 2000, Wren et al., 2000, Date et al., 2000) and in humans (Arvat et al., 2000, Takaya et al., 2000, Peino et al., 2000) showed that exogenously supplied ghrelin caused GH release. Ghrelin, as with the synthetic GHS, was found to affect GH release in humans in a dose dependent manner with the GH level returning to pretreatment levels about 1 hr after a single injection treatment (Seoane et al., 2000, Peino et al., 2000). Both ghrelin and des-Gln^{14} ghrelin showed similar abilities to stimulate GH release (Hosoda et al., 2000). However, removal of the octanoylate group on Ser-3 abolished GH secretion *in vitro* and *in vivo* (Bednarek et al., 2000).

The increase in serum growth hormone concentration is transient in nature after treatment with either GHS (reviewed by Bowers, 1999) or ghrelin (reviewed by Kojima et al., 2000). In addition to degradation of the ligand itself, this response has been

proposed to be the result of modulation of the entire GH/IGF-I axis, in which feedback results in attenuation of the response (see above). Further support for this mechanism attenuating the pituitary responsiveness to these GHSs is the down regulation of GHSR expression in the rat pituitary following administration of bovine GH (Bennett et al., 1997). Therefore, it may be concluded that administration of a GHS would not interfere with the normal pulsatility of GH secretion from the pituitary.

It appears that the response of the animal to GHS administration would be to stimulate the GH/IGF-I axis, which as described above, should translate into an increase in somatic growth. In fact, exogenously supplied GHS or ghrelin were found to significantly increase weight gain in humans, mice, rats and pigs (Bowers et al., 1984; McDowell et al., 1995; Phung et al., 2000; Tschop et al., 2000). The identification of increased circulating IGF-I concentrations in chronically treated animals, lead to the conclusion that this was the result of activation of the GH/IGF-I axis in the animals (Hickey et al., 1997; Svensson et al., 1998). Additionally, the improved feed conversion efficiency of treated swine, further indicated the effects were mediated in a GH dependent manner (Phung et al., 2000).

The conclusion that the effects were GH-dependent has been challenged by recent findings with ghrelin (Tschop et al., 2000; Wren et al., 2000) and to a lesser extent the GHRP-6 (Lall et al., 2001). Tschop et al., (2000) found that a single injection of ghrelin peripherally resulted in a significant shift in the metabolism of mice. They found that ghrelin caused a significant increase in the respiratory quotient, suggesting an increase in carbohydrate utilization and sparing of fat stores. This is in opposition to reported lipolytic effects of GH administration (reviewed by Etherton and Bouman, 1998). In fact,

exogenous ghrelin has been found to increase the fat mass of mice without altering lean or bone mass (Tschop et al., 2000). Although no studies have demonstrated the same effects with the syntheic GHS treatment, treatment of obese individuals with synthetic GHS suggest a similar mechanism of action. In obese individuals treated with MK-0677 for two months, there was an increase in lean body mass, but this was not matched with a change in fat tissue (Svensson et al., 1998). They suggested that lean mass accretion could be attributed to the GH response of the GHS, however this would also predict increased lipolysis, which was not observed in that study. This indicates, that in these obese individuals, the regulation of fat deposition by the GHS is independent of GH. The increased fat deposition following ghrelin administration may indicate a role for ghrelin and the GHS in the development of obesity in humans. However, both obese Caucasian and Pima Indians show reduced circulating ghrelin concentrations as compared to lean individuals of the same race (Tschop et al., 2001; English et al., 2002).

The reduced ghrelin concentration in the obese individuals was proposed to be the result of the perpetual positive energy balance, suggesting a role for ghrelin and GHS in the regulation of energy balance and feed intake. Exogenous ghrelin and GHRP-2 were found to significantly increase feed intake in mice and rats (Wren et al., 2001a; Tschop et al., 2002). Consistent with the role as an appetite-regulating factor, Cummings et al. (2001) found that ghrelin concentrations rise just prior to meal initiation in humans. Additionally, increased circulating ghrelin concentrations were observed in dieting obese individuals (Cummings et al., 2002). Evidence indicates that either ghrelin or GHS are able to activate the neuropeptide Y (NPY) and agouti-regulated peptide (AGRP) containing neurons of the hypothalamus (Dickson and Luckman, 1997; Kamegai et al.,

2001; Tschop et al., 2002). These data indicate that secretion of ghrelin by the stomach is acting in the hypothalamus as a long-term regulator of feed intake and nutrient partitioning. This is supported by the significant reduction in circulating ghrelin concentrations following gastric bypass (Cumming et al., 2002). In contrast to mammals, intracerebroventricular (ICV) injection of ghrelin into chickens caused a reduction in feed intake (Furuse et al., 2001). However, in this study, both the type (rat) and the mass (microgram quantities) of ghrelin may have resulted in abnormal findings. Further investigation is required to elucidate the mechanism of action of the GHS and ghrelin in the chicken.

Further implications for GHS in human health are suggested by their involvment in cancer development and cardiovascular health. The expression of the GHSR in pituitary adenomas (Korbonits et al., 1998; Kim et al., 2001), breast cancer (Cassoni et al., 2001), lung carcinoma (Ghe et al., 2002) and prostate cancer (Jeffery et al., 2002) cell lines suggest a role for GHS in the regulation of growth and development of cancerous tissue. Treatment of both hepatoma (Murata et al., 2002) and lung carcinoma cells (Ghe et al., 2002) with GHS and ghrelin increased proliferation of these tumor cells. The expression of ghrelin along with its receptor in the cancer cells indicates a potential autocrine/paracrine role for ghrelin in the growth of tumors *in vivo* (Jeffery et al., 2002).

Expression of the GHSR (Bodart et al., 1999) and GHS binding in myocardial tissues (Papotti et al., 2000) indicates a role for the GHS and ghrelin in the growth and development of cardiac function. The effects of the GHS on cardiac function have been shown both *in vitro* and *in vivo*. Locatelli et al., (1999) found that Hexarelin pretreatment counteracts ischemic damage in perfused rat hearts. This effect was demonstrated in both

intact and hypophysectomized animals, indicating that the effects were independent of GH. Additionally, in the pacing induced chronic heart failure model, GHS treatment improved ventricular function and myocyte contractile properties (King et al., 2000). Recently, chronic ghrelin treatment was found to improve the hemodynamic parameters, such as reducing the cardiac afterload and increasing cardiac output without altering heart rate (Nagaya et al., 2001).

Investigations into the effects of the GHS and ghrelin in the chicken have been very limited to date. However, identification of the GHSR cDNA sequence (Toogood et al., 1999) and identification of the GH releasing potential of the GHS (Geris et al., 2001) in the chicken have provided evidence of an active mechanism. To date, the endogenous ligand, ghrelin, has yet to be identified in the avian species. However, the identification of ghrelin immunoreactivity in the chicken pituitary indicates the presence of the ghrelin-like peptide in the chicken (Ahmed and Harvey, 2002). However, this study used a polyclonal anti-rat ghrelin antibody, and therefore did not conclusively show that the immunoreactivity was not simply the interaction with a separate octanyolated protein. Identification of the genomic or mRNA sequence of the ghrelin-like peptide will support an intact ghrelin-GHSR mechanism in the chicken.

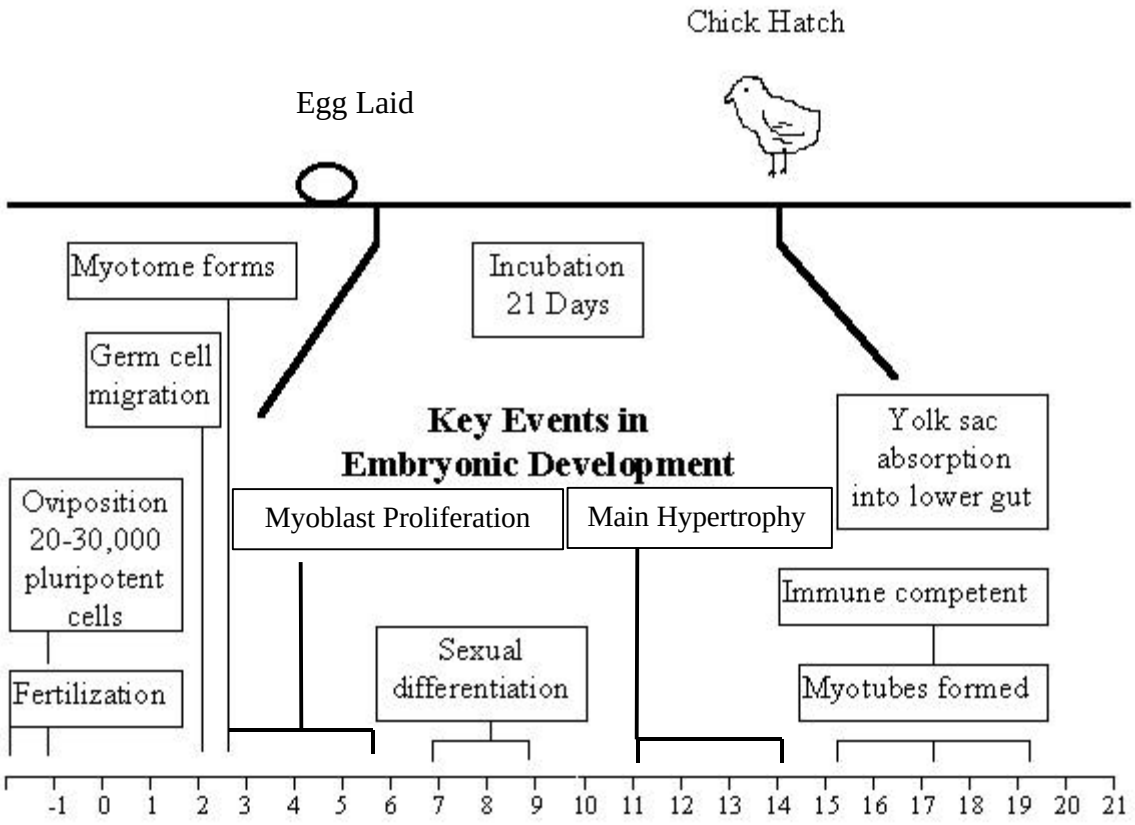


Figure I1 – Key Events of Embryonic Development

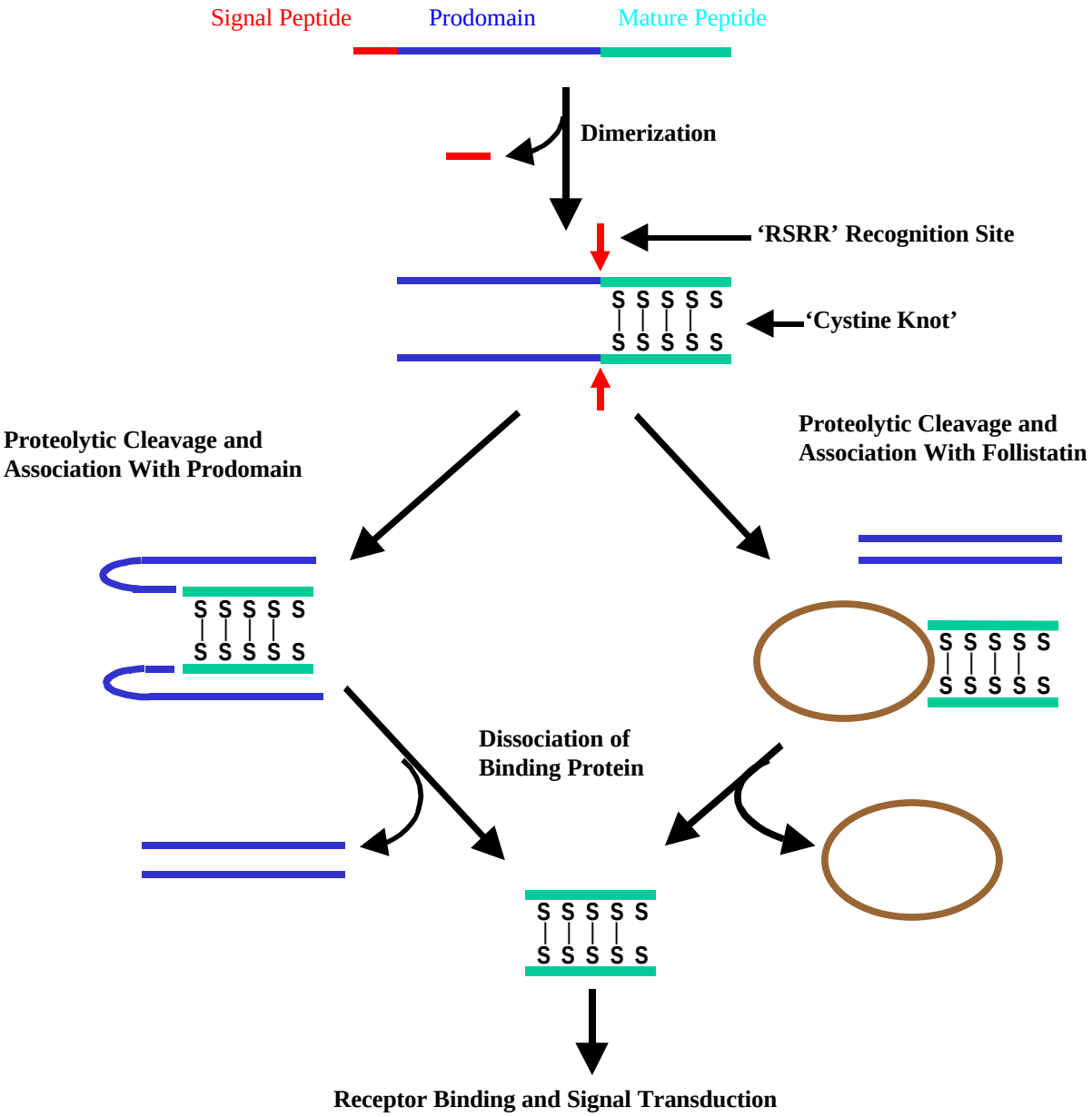


Figure I2 – Activation and Regulation of TGF-β (i.e. myostatin) Family Members.

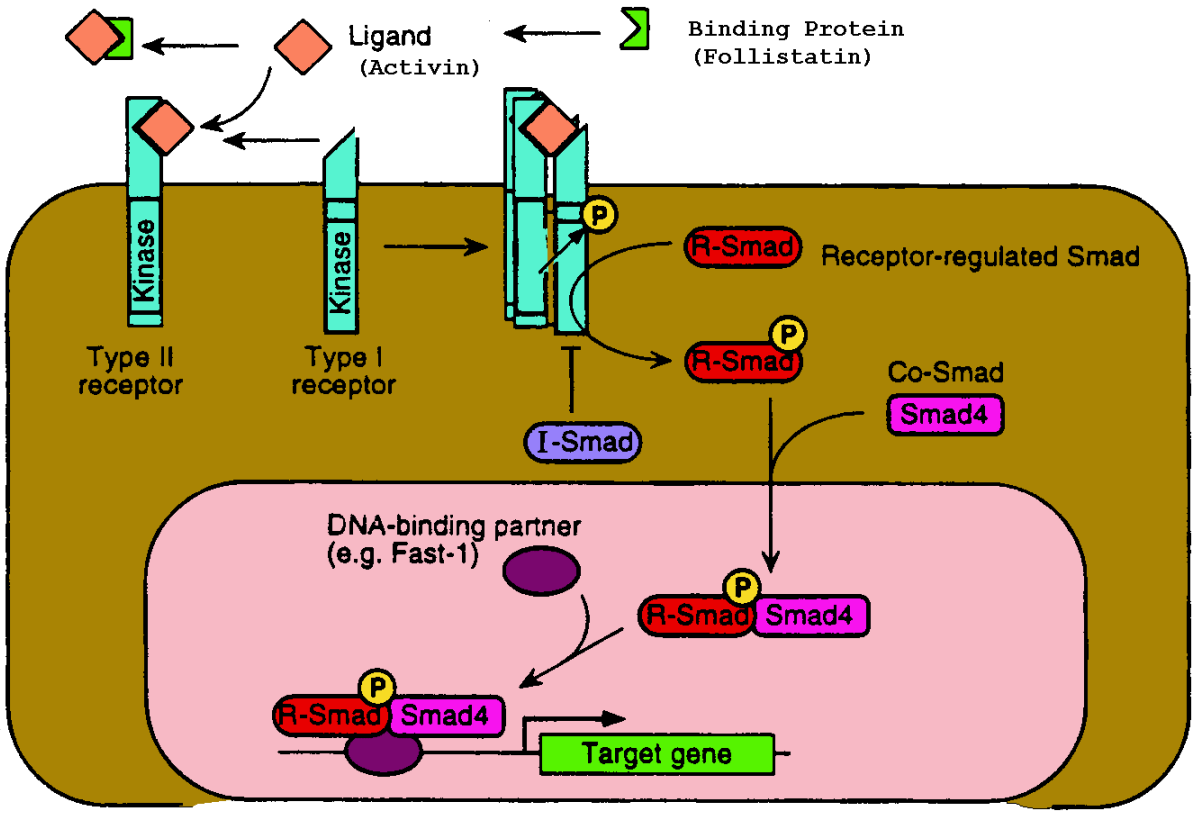


Figure I3 – TGF-β Signaling (Adapted from Massague, 1998)

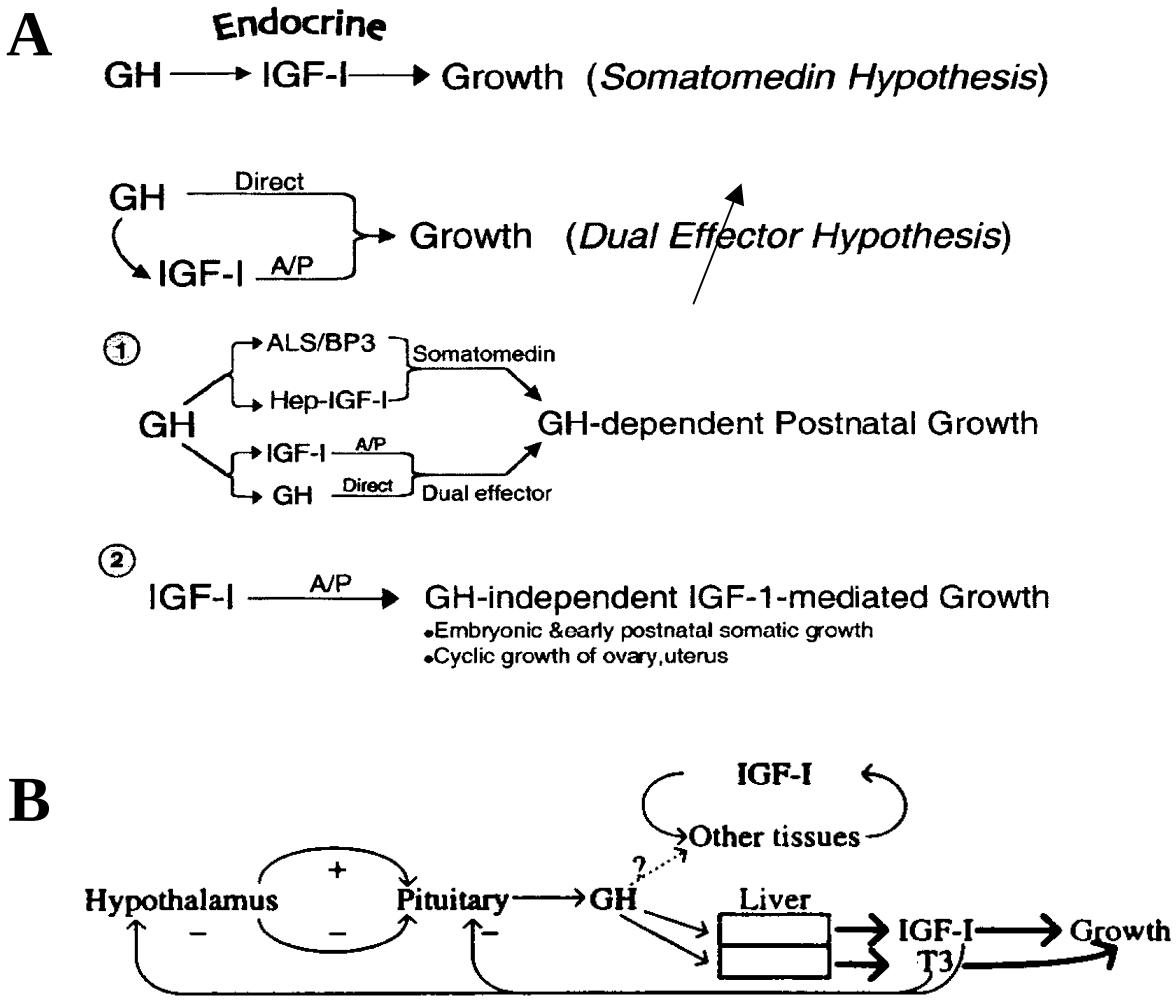


Figure I4 – A) Evolution of understanding of the GH/IGF-I Axis (from Le Roith et al., 2001) B) Diagram of the GH/IGF-I in Avians (A/P=Autocrine/Paracrine, ALS/BP3= Acid-Labile Subunit/Binding Protein 3) from Scanes, 1997).

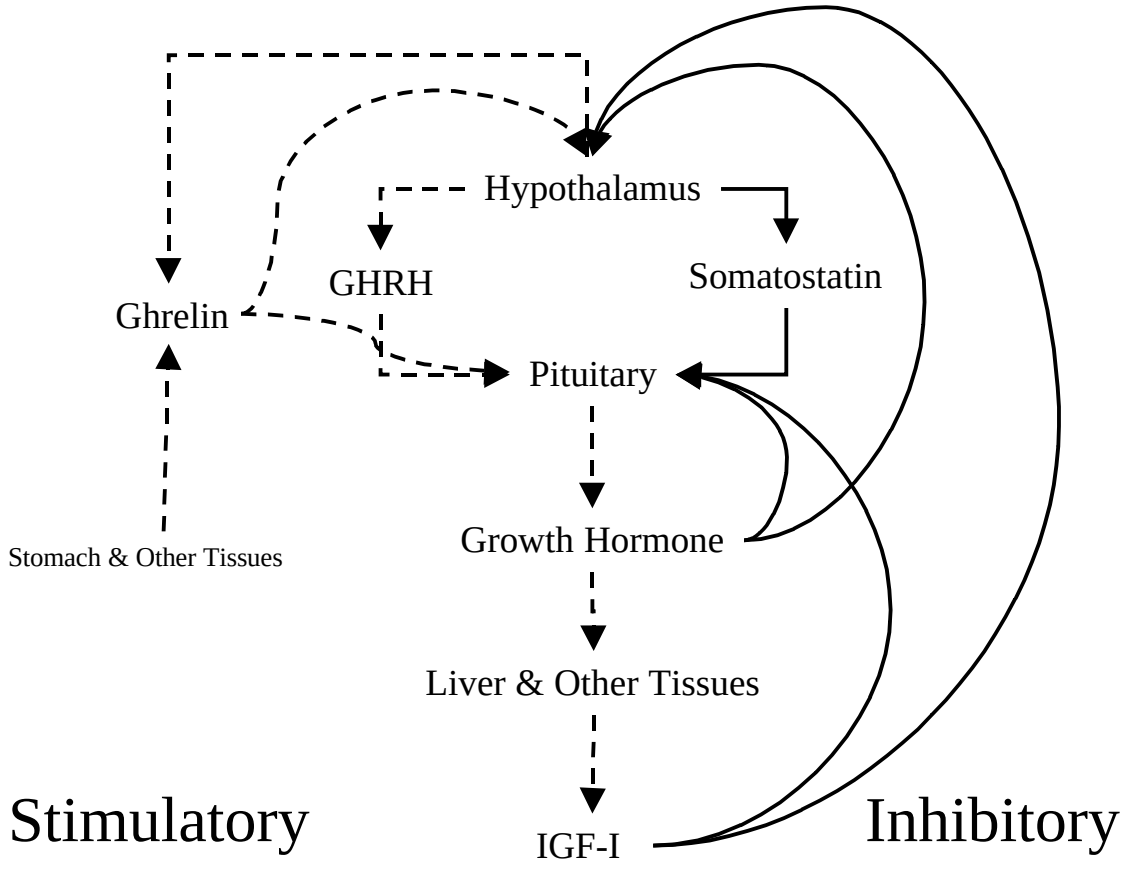


Figure I5 – Regulation of Growth Hormone Secretion

1977 Tyr-D-Trp-Gly-Phe-Met-NH_2 [D-Trp^2]MetEKNH_2

↓

1981 Tyr-D-Trp-Ala-Trp-D-Phe-NH_2

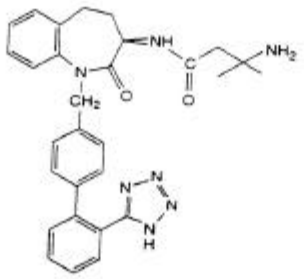
1984 His-D-Trp-Ala-Trp-D-Phe-NH_2

His-D-Trp-Ala-Trp-D-PheLys-NH_2 GHRP-6

↓

1991 Ala-His-DβNal-Ala-Trp-D-Phe-Lys-NH_2 GHRP-1

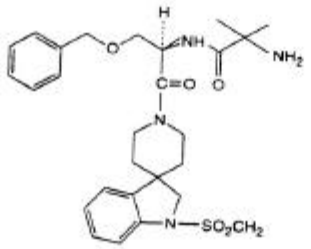
1992 His-D2MeTrp-Ala-Trp-D-Phe-Lys-NH_2 HEXARELIN



L-692,429 (MK-0751)

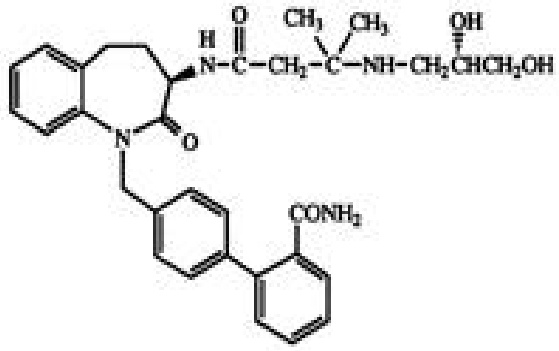
1993 D-Ala-DβNal-Ala-Trp-DPhe-Lys-NH_2 GHRP-2

1995

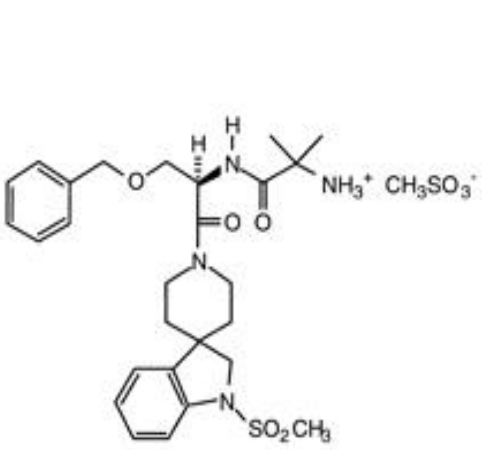


L-163,191 (MK-0677)

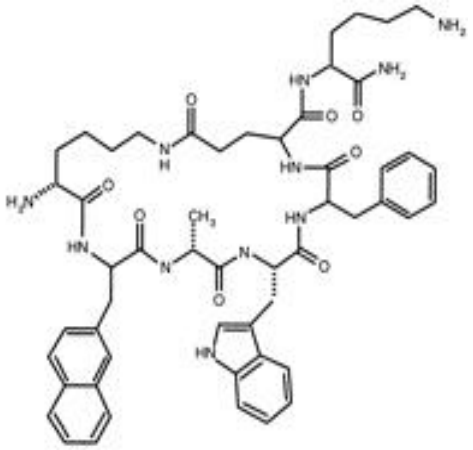
Figure I6 – Development of the Peptide and non-peptide Growth Hormone Secretagogues (from Muller et al., 1999)



Benzolactam Derivative (L-692,429)



Spiroindane Derivative (MK-0677)



Isonipecotic Derivative (G-7203)

Figure I7 – Representative Structure of each class of non-peptidyl Growth Hormone Secretagogue (adapted from Smith et al., 1997).

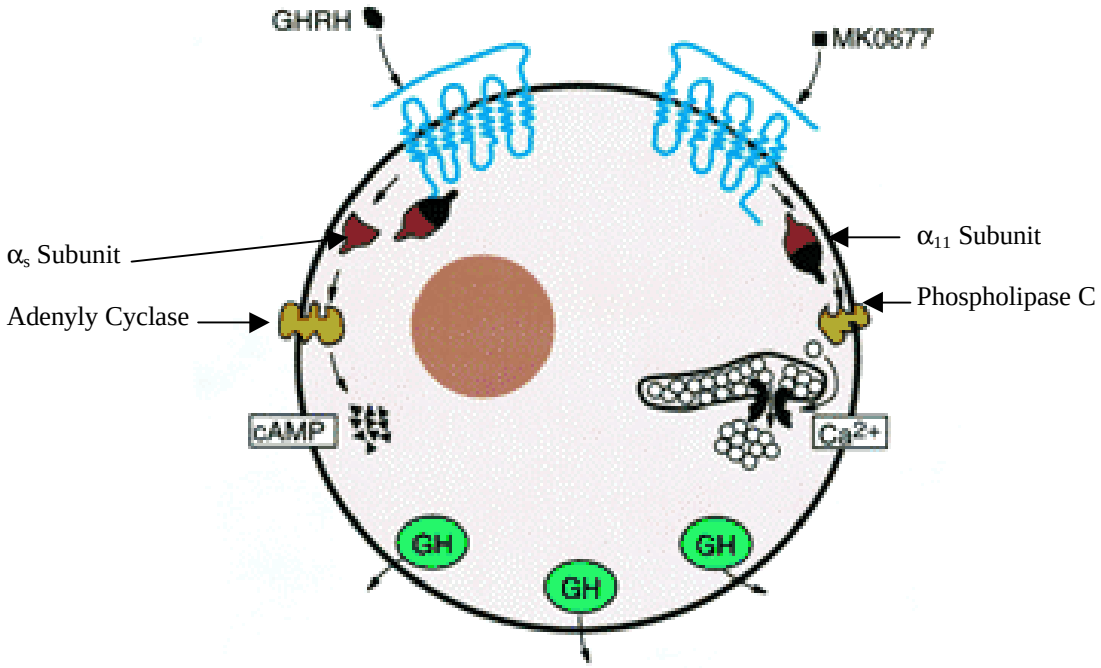


Figure I8 – Divergence of Signal Transduction by the Growth Hormone Releasing Hormone (GHRH) and the Growth Hormone Secretagogue (MK-0677). (From Smith et al., 1997)

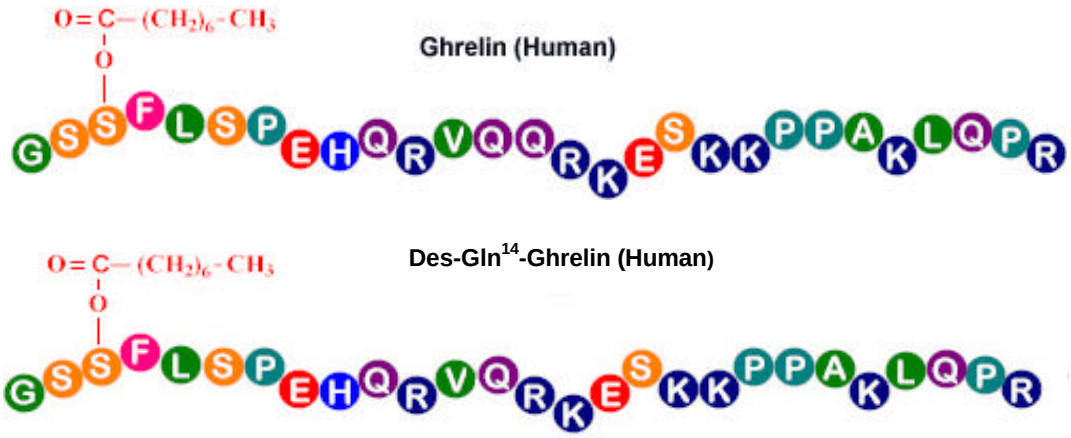


Figure I9 – Amino Acid Sequence of Human Ghrelin and des-Gln^{14}-Ghrelin showing the Location of the Octanoylate Residue on Amino Acid Three.

Chapter 1

In Ovo Administration Of Myostatin Antagonist Alters Postnatal Growth And Development Of The Broiler Chicken

INTRODUCTION

Myostatin (also known as Growth/Differentiation Factor 8), a member of the Transforming Growth Factor-β (TGF-β) family, is a negative regulator of skeletal muscle growth (Lee and McPherron, 1999). Targeted disruption of the myostatin gene in the mouse results in a 200-300% increase in muscle mass compared to that of normal littermates (McPherron, et al., 1997). In addition, the 'double muscle' phenotype in the Piedmontese and Belgian Blue breeds of cattle are associated with alterations in the myostatin gene (McPherron and Lee, 1997, Kambadur et al., 1997). Attenuation of the myostatin gene results in both hyperplasia and hypertrophy of muscle fibers (McPherron et al., 1997).

As with other members of the TGF-β family, myostatin is translated in a propeptide form, in which the cleavage of the N-terminus leads to the biologically active form of these growth factors (Sharma et al., 1999). The biologically active domain, the carboxyl terminal region, is highly conserved among mammalian and avian species suggesting a conserved function among these species (McPherron and Lee, 1997). Lee and McPherron (2001) have shown the ability of the C-terminal region to form dimers, which in turn bind to the activin type II receptors. This binding is inhibited by the action of the activin binding protein, follistatin, and to a lesser extent by the propeptide form of myostatin. Activation of myostatin begins with the removal of the N-terminus, i.e. the pro domain, which is able to bind to the C-terminal to inactivate myostatin signaling (reviewed by Sharma et al., 2001). Transgenic mice over expressing the pro domain of

myostatin show a significant increase in muscle hypertrophy, as a result of attenuated myostatin signalling (Yang et al., 2001).

In chicken embryonic development, primary muscle fiber formation occurs on embryonic day 7 and secondary muscle fiber formation occurs between embryonic days 7 and 18 (Feredette and Landmesser, 1991). Kocamis et al. (1999) found that myostatin expression during chick embryonic development increased on embryonic day 7 in the thoracic half and on embryonic day 17 in the *pectoralis* muscle, approximately overlapping with the timing of muscle fiber formation events. Additionally, they demonstrated a reduction in expression just prior to hatch when muscle fiber formation is nearly complete.

At present, ~80% of the broiler industry in America has begun to use *in ovo* vaccination against Marek's disease (Ricks et al., 1999). Therefore, the timing of vaccination provides an avenue for treatment with growth factors to increase poultry production. Previous work in our lab has shown that *in ovo* administration of growth factors is an effective method to modulate broiler growth and development (Kocamis et al., 1998). In the current study, we are evaluating the effect of *in ovo* administration of a myostatin antagonist (MA) on post hatch muscle growth and feed conversion efficency in the broiler. We hypothesize that the *in ovo* attenuation of myostatin will increase muscle growth without increasing the feed requirement for the additional muscle growth.

MATERIAL AND METHODS

Experimental Groups

Fertilized eggs (Ross x Ross) were obtained from Rocco Enterprises (Harrisonburg, VA) and placed into a Buckeye incubator/hatcher (temperature 37 ± .5 C, humidity 86 to 87%). Preliminary studies were conducted to determine both the optimum dosage and timing of administration of the myostatin antagonist. The results of these studies showed that 600 ng of myostatin antagonist administered between Days 15 and 20 of embryonic development showed the maximal response in terms of postnatal broiler growth. Thus, we selected Day 15 and Day 18 of embryonic development as treatment days. Eggs were injected once with 100 μL of vehicle (10 mM Acetic Acid, 0.1% BSA) or 100 μL of vehicle containing 600 ng of myostatin antagonist (MA) (Metamorphix, Inc., Baltimore, Md). The single injection was administered on either Day 15 or Day 18 of incubation. In addition to the vehicle injected control groups, an uninjected control group was kept to assess the effect of the *in ovo* injection procedure. The injection procedure has been previously described (Kocamis et al., 1998). Briefly, eggs were removed from the incubator, and the blunt end was sterilized with 70% ethanol. A dental drill bit was used to make a hole through the shell, without penetrating the chorio-allantonic membrane. A 22-gauge needle was used to puncture the membrane and deliver the 100 μL of vehicle or vehicle containing 600 ng of MA. The hole was sealed with an adhesive sticker before replacing in the incubator. A total of 1078 eggs were set in the incubator and assigned to the following groups: uninjected control (226 eggs), Day

15 Vehicle Injected (213 eggs), Day 15 MA Injected (213 eggs), Day 18 Vehicle Injected (213 eggs) and Day 18 MA Injected (213 eggs).

At hatch, the birds were vaccinated for Marek's Disease (200 μL s.c., Murray McMurray Hatchery, Des Moines, IA) and separated by feather sexing. Four replicates (pens) of each of the five treatments (Two MA Treated, Two control treated, one uninjected control) were randomly assigned to a total of 20 pens. In each pen, a total of 48 birds (24 male +24 female) were assigned.

Housing and Data Collection

The animals were housed in open floor pens in a temperature controlled building with a 24-hr lighting regime. For the first three weeks, the birds were fed a standard starter ration consisting of 21.2% crude protein and 3080 kcal/kg. Starting at three weeks, this was replaced by the grower ration consisting of 19.5% crude protein and 3124 kcal/kg. Throughout the study, the birds were given ad libitum access to feed and water. To assess the effects on growth, pen weights were recorded on a weekly basis up to six weeks of age. Additionally, the feed was weighed in and the orts determined once a week to measure feed intake and feed conversion efficiency.

At six weeks of age, 120 birds (12 males and 12 females per treatment) were randomly selected for analysis of muscle growth and quality. For each bird, the live weight was recorded, then they were slaughtered and sectioned to determine the whole breast (muscle + rib cage), leg and thigh, heart, and liver weights. The shank length was determined using calipers to measure along the medial side of the tibia. From each

treatment group, Pectoralis major muscles (6 male and 6 female) were collected, vacuum packed and stored at –20ºC for shear force analysis.

Shear force analysis was conducted as previously outlined (Iqbal et al., 1999). Briefly, the Pectoralis major muscles were thawed overnight at 4ºC, weighed, then cooked to an internal temperature of 70ºC on a Farberware Smokeless Indoor Grill (Farberware, Inc., Bronx, NY). The cooked muscle was cooled to room temperature, and three to four 1.27-cm diameter cores were made through the thickest portion of the muscle perpendicular to the fiber orientation. Warner-Bratzler shear value was determined using an Instron Universal Mechanical Testing Machine (Model TM, Instron Corp., Canton, MA). A Warner-Bratzler appratus was attached and output was aquired with a DT 2805 data acquisition board (Data Translation, Marlboro, MA). The data was processed with the HP-VEE software package (Hewlett Packard Co., Loveland, CO).

Statistical Analysis

Analysis of variance was performed using the GLM procedure of SAS® (Cary, NC). Least significant difference test was used to compare means. Average pen weight and feed conversion efficiency means were compared between treatments within each treatment day. For all other variables, same sex means were compared within the treatment day (MA vs. Vehicle).

RESULTS

Hatch, Body Weight Gain and Feed Conversion

Hatch data is shown in Table 1-1. Neither the hatch rate nor the number of dead embryos was changed by vehicle or MA administration. At hatch, the average bird

weight was similar for all treatment groups (Figure 1-1), and although the MA treated groups appeared to be larger at week 4 and 5, there was no significant difference in the average growth rate between the MA and vehicle treated birds (Figure 1-1). There was not effect of day of injection on the cumulative feed conversion efficiency at six weeks (Figure 1-2).

Muscle Characteristics

At six weeks of age, MA treatment on Day 15 or 18 had no effect on any of the body weight measurements made for the male birds (Figure 1-3, 1-4, 1-5, 1-6). Similarly, Day 18 treatment did not significantly altered the six-week old female broiler body weight measurement or shank length. However, Day 15 administration increased the breast wt. (14%, $P<0.05$, Figure 1-4) and shank length (7%, $P<0.01$, Figure 1-6) in the female birds. Additionally, there was a trend to increase the live wt. (8%, $P=0.093$, figure 1-3) and leg and thigh wt. (10%, $P=0.079$, figure 1-5) at six weeks of age. MA did not change the shear force value for either the male or female *Pectoralis major* muscle (Figure 1-7).

DISCUSSION

We have found that MA administration on Day 15, but not Day 18, of embryonic development significantly altered the post hatch growth and development of female broiler chickens. However, similar results were not found in the male bird. This may indicate potential sexual dimorphic effects with respect to myostatin activity in the chicken. Henry and Burke (1998) found that female broilers had a reduced number of

myofibers per area in the *Pectoralis superficialis* at embryonic day 20 compared males. This may indicate a mechanism limiting the proliferation of myoblasts in the female embryo. Since myostatin has been shown to limit myoblast proliferation *in vivo* (Thomas et al., 2000), changes in the sensitivity to myostatin may lead to differences during embryonic development. Thus, the increased effect shown on muscle growth in the female broiler may indicate such a relationship exists in the chicken. However, the current study did not investigate the cause of the increased breast weight (hyperplasia vs. hypertrophy), which would reveal the nature of the effects of MA treatment on the female muscle growth and development.

From a production prospective, the sexually dimorphic growth patterns of male and female broilers is well known. We found that *in ovo* MA treatment on Day 15 increased the breast muscle weight of the female broiler at harvest. This may provide a method to lower the discrepancy between male and female broilers and increase total productivity. Additionally, the increased muscle mass of female birds did not effect tenderness of the *Pectoralis muscle* (Figure 1-7). This may indicate the ability of *in ovo* MA treatment to be an effective method to increase muscle growth of the broiler with out altering palatability.

In addition to the sex differences in response to the MA, we also observed an increased response with Day 15 administration versus Day 18 administration. Kocamis et al. (1999) found a significant increase in the expression of myostatin mRNA on embryonic day 17 in the skeletal muscle. This may be an indication of the dose dependence of the response during development. The increased expression of myostatin observed on Day 17 of embryonic development (Kocamis et al., 1999) would indicate a

greater concentration of the protein present on day 18. This may indicate the requirement for a greater amount of MA to be administered on Day 18 to observe a similar response.

In female broiler chickens, we observed a greater increase in the breast muscle than was reflected in the leg and thigh muscle (Figures 1-3 and 1-4). The breast muscle of a chicken contains a greater proportion of white muscle fibers as compared to the leg and thigh muscles. Carlson et al. (1999) showed that the highest myostatin expression was found in muscles with the highest levels of white muscle fibers in mice. Therefore, the greater increase in the mass of the breast muscle (14%) as compared to the leg and thigh muscles (10%) may reflect a similar relationship between the development of different muscle types in the chicken. Further investigation is required to assess the distribution of muscle fiber types in the muscles of the chickens in response to MA administration.

Myostatin may also interact with other members of the TGF-β family and/or binding proteins of this family to regulate overall growth and development. In the current study, in addition to the increase in muscle weight, there was also an increase in the shank length of the female birds treated on Day 15, indicating a potential interaction between the MA and other members of the TGF-β family. Evidence for such an interaction is demonstrated between follistatin, an activin binding protein, and myostatin in the regulation of muscle growth. Over expression of follistatin in mice led to a significant increase in the muscle mass of the mice (Lee and McPherron, 2001).

Although no gross abnormalities were noted in the current study, this may indicate a change in the mechanical qualities of the bones in the MA treated group. The

potential interaction between the MA and bone growth warrants further investigation because of potential implications to the poultry industry.

In the current study, we investigated the effects of *in ovo* administration of a MA on the post hatch growth of broiler chickens. We found that administration on Day 15 of embryonic development significantly increased the breast wt (14%) and shank length (7%) and showed a tendency to increase the live wt (8%) and leg and thigh weight (10%) of the female chickens without changing feed conversion efficiency. Coupling this treatment with existing *in ovo* vaccination techniques provides a feasible method of enhancing growth. Additionally, the enhanced effects in females could provide a method to lessen the gender discrepancy in mixed-sex broiler production.

Table 1-1 - Hatchability

Treatment[1]	Control	D15 Veh	D15 MA	D18 Veh	D18 MA	Total
Eggs Treated	226	213	213	213	213	1078
Number Infertile	8	7	5	3	7	30
Fertile Dead	21	13	18	14	25	91
Hatch	197	193	190	194	180	954
Male	96	94	95	95	98	478
Female	101	99	95	99	82	476
% Infertile	3.5%	3.3%	2.3%	1.4%	3.3%	2.8%
% Dead	9.3%	6.1%	8.5%	6.6%	11.7%	8.4%
% Hatch[2]	90.4%	93.7%	91.3%	92.4%	87.4%	91.0%

1- Treatment Groups: Control- Uninjected Control, D15 – Injection on Day 15, D18 – Injected on Day 18, Veh – Vehicle Injected, MA – Myostatin Antagonist Injected

2- % Hatch = No Live Hatched/ (No eggs injected – No infertile Treated)

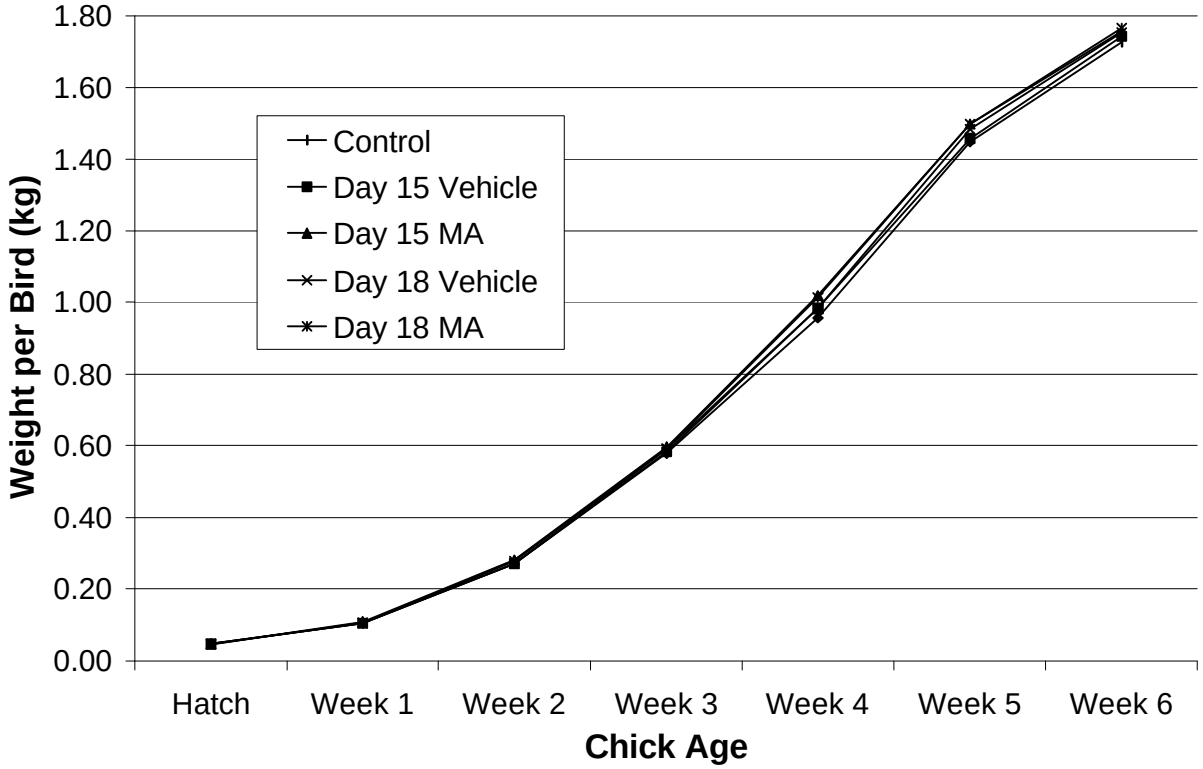


Figure 1-1- Average mixed-sex broiler weight measured as pen weight at end of each week (n = 4 for each treatment) (Treatment Groups Control – uninjected control, D15 Vehicle – Vehicle injected on Day 15, D15 MA – Myostatin Antagonist injected on Day 15, D18 Vehicle – Vehicle injected on Day 18, D18 MA, Myostatin Antagonist injected on Day 18).

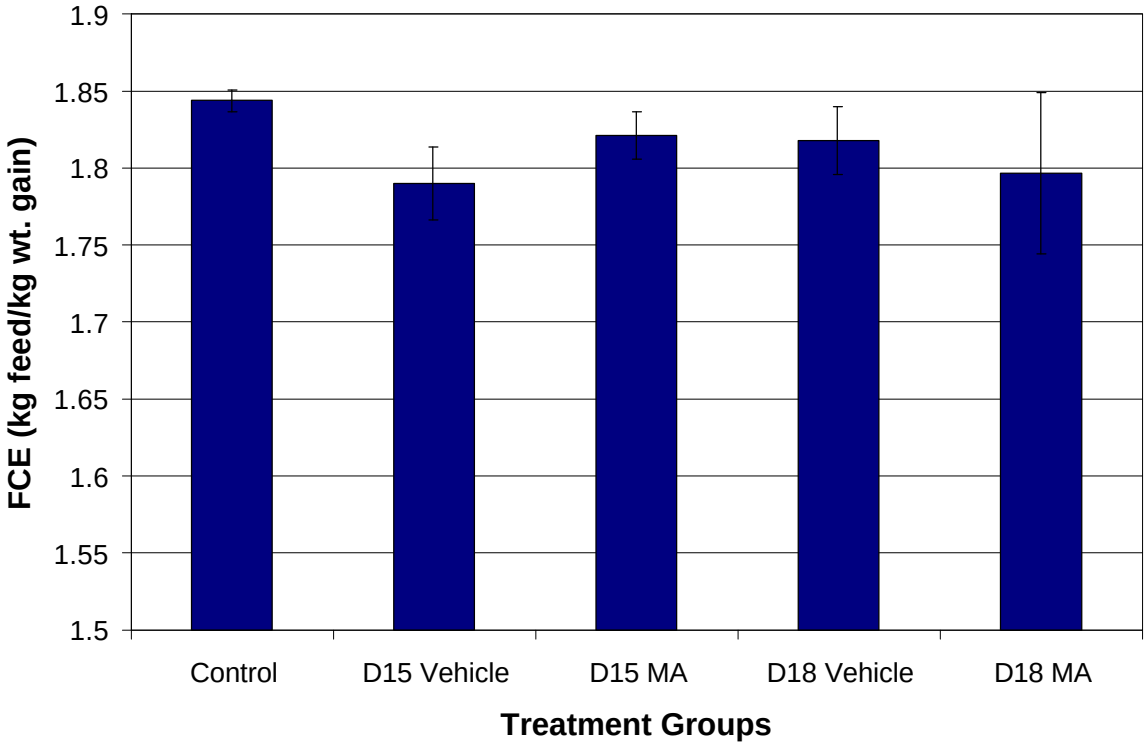


Figure 1-2 – Effect of *in ovo* MA administration on average mixed sex cummulative feed conversion efficiency (mean ± SEM; n=4 for each treatment) at six weeks of age. (Treatment Groups Control – uninjected control, D15 Vehicle – Vehicle injected on Day 15, D15 MA – Myostatin Antagonist injected on Day 15, D18 Vehicle – Vehicle injected on Day 18, D18 MA, Myostatin Antagonist injected on Day 18).

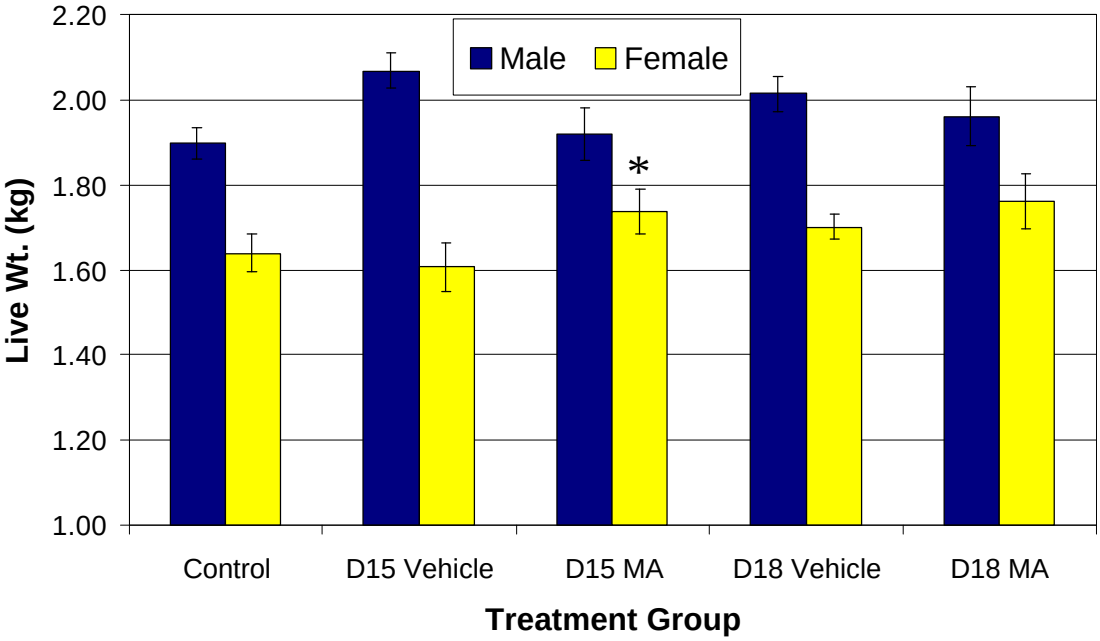


Figure 1-3 – Effect of *in ovo* MA administration on live weight of male and female broilers at six weeks of age (mean ± SEM; n=12 for sex within each treatment, $p < 0.10$ compared to same sex control). (Treatment Groups Control – uninjected control, D15 Vehicle – Vehicle injected on Day 15, D15 MA – Myostatin Antagonist injected on Day 15, D18 Vehicle – Vehicle injected on Day 18, D18 MA, Myostatin Antagonist injected on Day 18.)

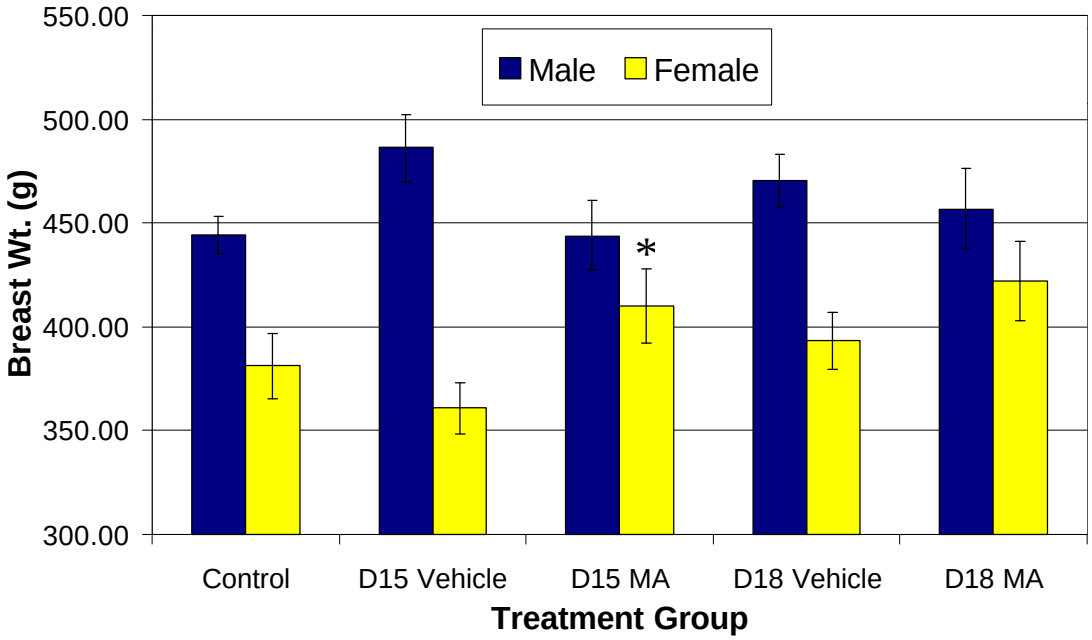


Figure 1-4 – Effect of *in ovo* MA administration on breast weight of male and female broilers at six weeks of age (mean ± SEM; n=12 for sex within each treatment, $p < 0.05$ compared to same sex control). (Treatment Groups Control – uninjected control, D15 Vehicle – Vehicle injected on Day 15, D15 MA – Myostatin Antagonist injected on Day 15, D18 Vehicle – Vehicle injected on Day 18, D18 MA, Myostatin Antagonist injected on Day 18.)

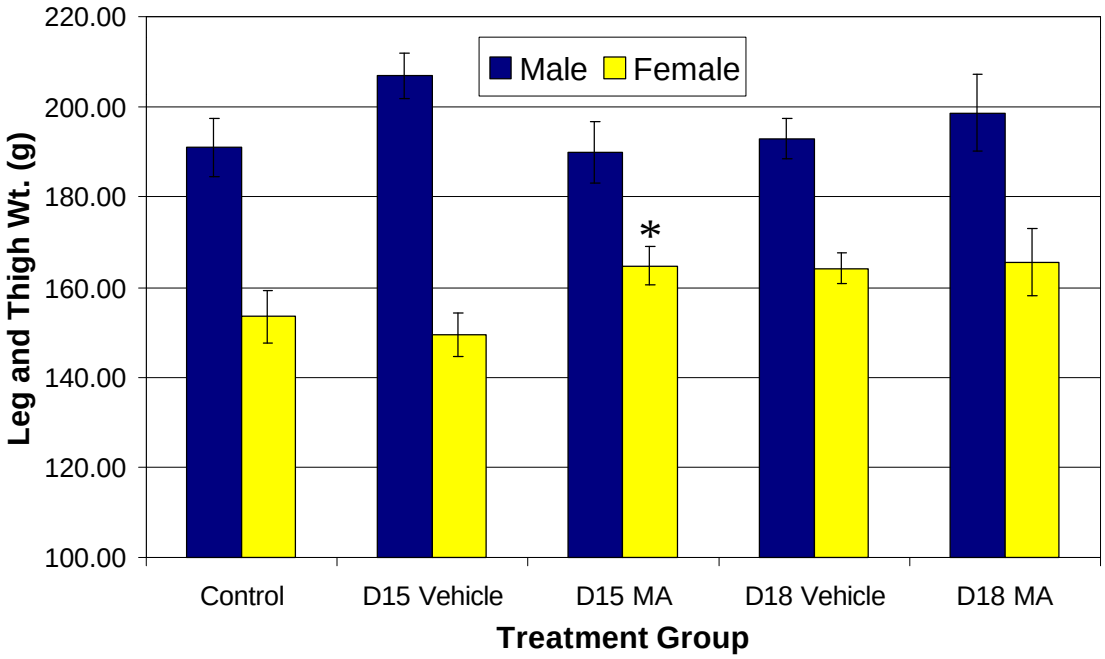


Figure 1-5 – Effect of *in ovo* MA administration on Leg and Thigh weight of male and female broilers at six weeks of age (mean ± SEM; n=12 for sex within each treatment, *- $p < 0.10$ compared to same sex control). (Treatment Groups Control – uninjected control, D15 Vehicle – Vehicle injected on Day 15, D15 MA – Myostatin Antagonist injected on Day 15, D18 Vehicle – Vehicle injected on Day 18, D18 MA, Myostatin Antagonist injected on Day 18.)

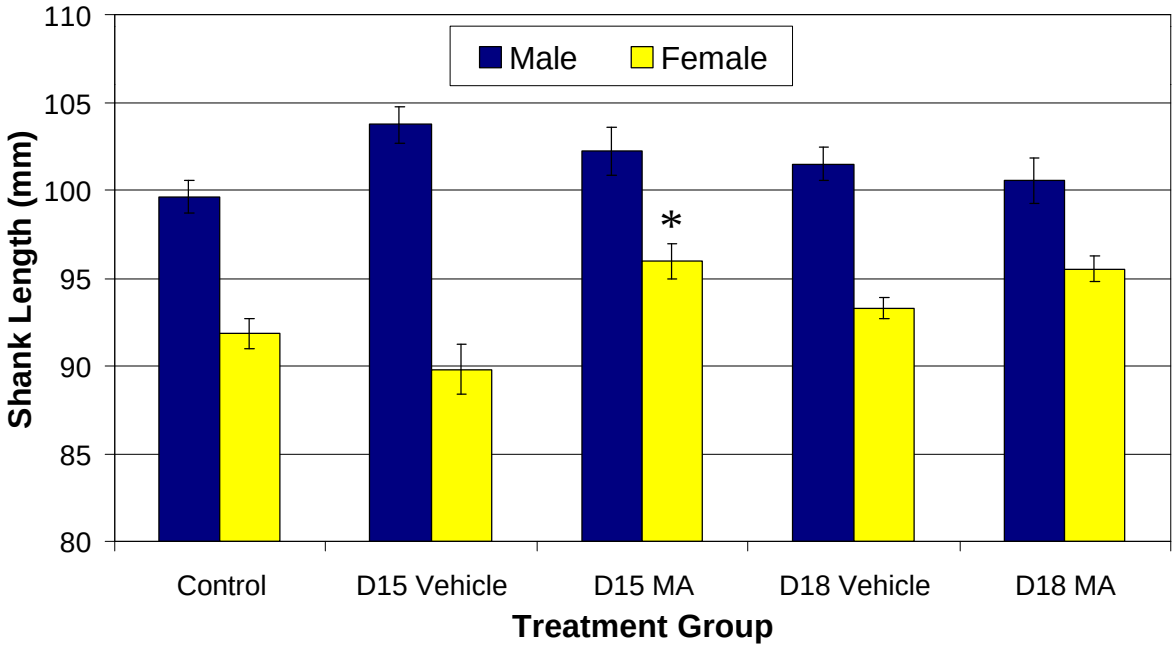


Figure 1-6 – Effect of *in ovo* MA administration on Shank length of male and female broilers at six weeks of age (mean ± SEM; n=12 for sex within each treatment, *- $p < 0.01$ compared to same sex control). (Treatment Groups Control – uninjected control, D15 Vehicle – Vehicle injected on Day 15, D15 MA – Myostatin Antagonist injected on Day 15, D18 Vehicle – Vehicle injected on Day 18, D18 MA, Myostatin Antagonist injected on Day 18.)

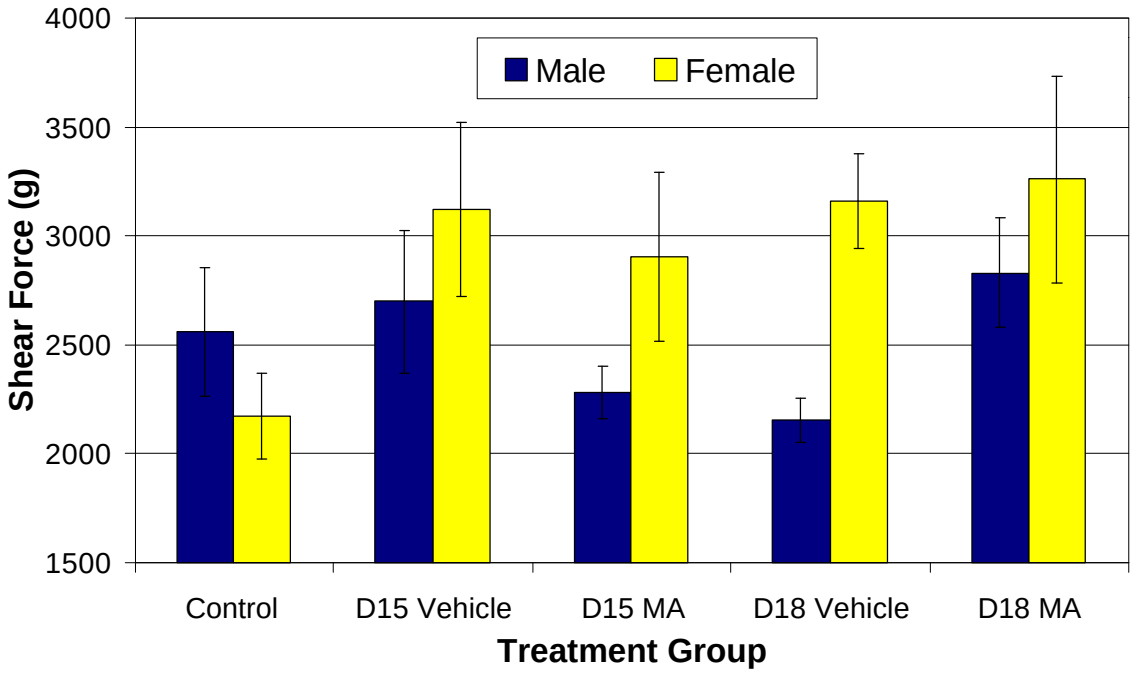


Figure 1-7 – Effect of *in ovo* MA administration on *Pectoralis major* shear force of male and female broilers at six weeks of age (mean ± SEM; n=6 for sex within each treatment). (Treatment Groups Control – uninjected control, D15 Vehicle – Vehicle injected on Day 15, D15 MA – Myostatin Antagonist injected on Day 15, D18 Vehicle – Vehicle injected on Day 18, D18 MA, Myostatin Antagonist injected on Day 18.)

Chapter 2

The Effects of *In Ovo* rhIGF-I Administration on Expression of the Growth Hormone Secretagogue Receptor (GHSR) During Chicken Embryonic Development.

INTRODUCTION

The secretion of growth hormone (GH) has been shown to be necessary for normal postnatal growth in avian species (Vasilatos-Younken and Scanes, 1991). The pulsatile release of GH by the anterior pituitary is primarily regulated by the interaction of a pair hypothalamic factors, Growth Hormone Releasing Hormone (GHRH, stimulatory) and Somatostatin (inhibitory) (for review see Muller et al., 1999). Recently, a group of synthetic peptides and non-peptidyl analogues, collectively referred to as the GH secretagogues (GHS), have been identified with the ability to induce GH secretion without altering the release of other anterior pituitary hormones (for review see Smith et al., 1997). It has also been demonstrated that GHS regulate the secretion of GH through the recently identified GHS receptor (GHSR; Howard et al., 1996), independent of the hypothalamic factors (Bowers et al., 1991)

The lack of detectable serum growth hormone until embryonic Day 17 (E17) (Harvey et al., 1979) and pituitary GH mRNA until E18 (McCann-Levorse et al., 1992) has led to the conclusion that early embryonic chicken development is GH-independent. However, Harvey et al. (2000) found GH-immunoreactivity and GH Receptor (GHR)-immunoreactivity throughout the peripheral tissues of E3, E6 and E7 chick embryos. Additionally, GH mRNA expression from whole embryos has been detected during this time period, prior to sotamotroph differentiation on E16 (Porter et al., 1995), suggesting an anterior pituitary independent role for GH during early embryonic development (Harvey et al., 2000, Murphy and Harvey, 2001). Expression of the GH regulated gene-1 (GHRG-1) in many, but not all tissues, on E8 shows active signaling through the GH-R during embryonic development, supporting autocrine/paracrine function of GH (Harvey

et al., 2001). Based on these recent findings, the mechanism (s) that regulates the expression and secretion of GH during early embryonic development has to be reevaluated. In the current study, we examined growth hormone secretagogue receptor expression in the whole embryo during early embryonic development, and in the *pectoralis* muscle during later embryonic development. Additionally, we have previously demonstrated that *in ovo* administration of recombinant human Insulin-Like Growth Factor-I (rhIGF-I) on E3 improved posthatch muscle development (Kocamis et al., 1998). Therefore, we assessed changes in the expression pattern of the GHSR in response to *in ovo* rhIGF-I administration on E3.

MATERIALS AND METHODS

Treatment and Sample Collection

Fertilized eggs (Cobb x Cobb) were obtained from Rocco Enterprises (Harrisonburg, VA) and placed into a Buckeye incubator/hatcher (temperature 37 ± .5 C, humidity 86 to 87%). *In ovo* administration of recombinant human IGF-I (rhIGF-I) and sample collection was performed as previously described (Kocamis et al., 1999). Briefly, eggs were injected once on embryonic Day 3 (E3) with 100 ng of rhIGF-I (R&D Systems, Minneapolis, MN) dissolved in 100 μL of 10 mM Acetic Acid and 0.1% BSA. Before injection, eggs were removed from the incubator, and the blunt end was sterilized with 70% ethanol. A dental drill bit was used to make a hole through the shell, without penetrating the chorio-allantonic membrane. A 22-gauge needle was used to puncture the membrane and deliver the 100 μL of vehicle or rhIGF-I. The hole was sealed with an

adhesive sticker before placing back in the incubator. Embryos and tissues were harvested in compliance with an approved West Virginia University Animal Care and Use Committee Protocol. All embryos were isolated and washed free of yolk, albumen and extra-embryonic membranes with sterile nuclease-free water. On E0 to E6, whole embryos (n=6 per day) were collected. Thoracic/abdominal halves of the embryos from the lumbo-sacral level to the neck without head were collected on each of E 7 and E 8 (n= 6 per day). *Pectoralis* muscle was collected on each of E 9 to E 20 (n= 4 per day).

RNA Isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from each sample following the TRI-Reagent (Molecular Research Center Inc., Cincinnati, OH) modification of the guanidine isothiocyanate/phenol-chloroform method originally described by Chomczynski and Sacchi (1987). The quality of the RNA was assessed by formaldehyde agarose gel electrophoresis with the visualization of the 18s and 28s rRNA bands. All RNA samples were stored at –80°C. Reverse transcription was performed with 2 μg of total RNA, 2 μg of random hexamer primers and Murine Maloney Leukemia Virus reverse transcriptase (MMLV-RT) (Promega, Madison, WI). Polymerase chain reactions were performed as previously outlined (Kocamis et al., 2001). Briefly, 2 μL of the reverse transcription reaction was added to a 50 μL of the PCR cocktail containing: 5 μL of 10x Taq buffer (1.5 mM $MgCl_2$ final concentration), 1 μL dNTPs (final concentration 0.2 mM each), 1 μL of sense and anti-sense primers (1 mM final concentation), 1 μL of Taq DNA Polymerase (Display Systems Biotech, Vista, CA) and 41 μL of nuclease free water. For the GHSR, touchdown PCR was used. This program consisted of a 5-min denaturation

step (94°C), followed by 5 cycles in which the initial annealing temperature of 69°C was reduced by 1°C per cycle, then 30 cycles in which the annealing temperature was 65°C. The reaction was concluded with a 10-min extension step at 72°C. For the 18s-rRNA the reaction was a standard PCR reaction. This program consisted of a 5-min denaturation step (94°C), followed by 25 cycles in which the annealing temperature was 55°C. Again the reaction was concluded with a 10-min extension step at 72°C. Gene specific primers were designed based on published sequences of the chicken putative GHSR (genbank accession # AJ309543) and chicken 18s rRNA sequence (genbank accession # AF173612) and were obtained from Gibco BRL Inc. (Grand Island, NY). For GHSR, the forward primer sequence was 5'-TCGCCATCTGCTTCCCTCTG-3' and the reverse primer sequence was 5'-GCACCGTGAGGCAGAATAC-3'. Alignment with the putative sequence published had a 262-bp PCR product corresponding to bases 125-387 of the putative chicken GHS-R. The forward sequence of 18S rRNA was 5'-CGGCGGCTTTGGTGACTCTA-3' and the reverse primer sequence was 5'-CGCCGGTCCAAGAATTTCAC-3'. The two primers produced a 697 bp product and corresponded to the bases 199-895 of the chicken sequence. All reactions were run on a PTC-200 thermocycler (MJ Research, Watertown, MA). A water control (no cDNA) reaction was run with each cocktail to assure that there was no contamination of the components of the reactions. The identities of the PCR products were verified by sequence analysis.

The amplified gene products were visualized by electrophoresis on 1.5% agarose gels stained with ethidium bromide (0.2 mg/mL). The gene products were photographed under UV light using a FluorChem^{TM} Imaging System (Alpha-Innotech Corp., San

Leandro, CA) and quantified by densitometric analysis of the photograph using FlourChem™ Software (Alpha-Innotech Corp., San Leandro, CA). The densitometric values for each gene product were normalized to densitometric values of the 18S rRNA housekeeping gene. Regression analysis was performed by the GLM procedure of SAS® (Cary, NC). Statements of significance were based on $P < 0.05$ unless otherwise noted.

RESULTS

The control regression equation for GHSR was $y = 0.141*day – (0.016*day^2) + (0.00058*day^3)$ ($r^2=0.25$, $P<0.01$). Figure 2-1a depicts the expression pattern for the GHSR during embryonic development. Expression was low on E0 and remained at about the same level through E4. There was a ~2.5 fold increase in expression on E5. Although GHSR expression fluctuated during the mid-embryogenesis, it remained fairly constant through E16 of incubation. Starting on E17, there was an increase in the expression, which was followed by the highest expression in the breast muscle on E19. The regression equation for the IGF-I treated group was $y = (0.378*day) – (0.047*day^2) + (0.0014*day^3)$ ($r^2=0.15$ $P<0.05$). The expression pattern is shown in figure 2-1b. As with the control group, we found low expression on E0 and that it remained low until E4. The expression of GHSR increased ~7 fold on E5 and continued to increase through E7. Expression was reduced on E8 and remained fairly constant through E16. Then, expression further increased on E17 and reached the highest level on E19 before reducing on E20.

Figure 2-2 shows the expression pattern of 18s rRNA during embryonic development. 18s rRNA was used as the housekeeping gene in the current study. For both the control and rhIGF-I treated groups, expression was constant throughout embryonic development.

DISCUSSION

In the current study, we investigated the expression of the GHSR during chicken embryonic development, with a particular focus on skeletal muscle. We found that expression was low during the early embryonic period (Figure 2-1). Harvey et al. (2000) found GH-immunoreactivity in most tissues and organs during this time of chicken embryonic development. GHRH responsiveness of developing chicken embryos has been shown to be very limited before E14 (Darras et al., 1994). Additionally, in chickens, unlike mammals, thyrotropin-releasing hormone (TRH) induces GH secretion from the pituitary (for review see Scanes et al., 1984). Identification of a cDNA corresponding the GHSR from a pituitary cDNA library (Toogood et al., 1999) and GHS's ability to increase GH secretion both directly in the anterior pituitary and through TRH in the hypothalamus (Geris et al., 2001) suggest the existence of a GHS-mediated mechanism of GH release in the chicken. This leads to the possibility that the GHSR is acting to regulate the expression of GH and GH may be functioning in an autocrine/paracrine fashion in the peripheral tissues during embryonic development.

In ovo administration of GH has been shown to inconsistently alter the post hatch muscle growth and development of chickens (Kocamis et al., 1999, Hargis et al., 1989). This may be a result of the autocrine/paracrine nature of the GH found in early

embryonic chicken tissues (Harvey et al., 2001). However, *in ovo* treatment with rhIGF-I on E3 was found to significantly increase breast weight of 6-week-old broilers (Kocamis et al., 1998). In the intact post-hatch bird, GH stimulated IGF-I secretion from the liver and other tissues is required for normal growth (reviewed by Scanes, 1997). We investigated the possibility that *in ovo* administration of rhIGF-I may have altered GH synthesis through the recently identified GHSR. We found that there was an increase in the expression of GHSR gene in the thoracic/abdominal section of the IGF-I administered embryos between E6-E8. In the chicken embryo, primary muscle fiber formation begins on E7 with secondary muscle fiber formation occurring between E7-E18 (Fredette and Landmesser, 1991). During this period of embryonic development, both GH immunoreactivity (Harvey et al., 2000) and GH-R mediated gene regulation (Harvey et al., 2001) have been found in the developing muscle tissues. This challenged the widely held tenant that early embryonic development is GH independent, and suggested the GH may be acting in an autocrine/paracrine manner to regulate embryonic muscle development. Thus, our finding suggests that IGF-I acts to increase the expression of the GHSR during primary muscle formation, which in turn may act to increase the expression of GH in the peripheral tissues (i.e. the muscle).

We found highest expression of the GHSR mRNA level on E19 in the *pectoralis* muscle, concommitant with the dramatic increase in the serum GH concentration during this time frame (Harvey et al., 1979). Although the contribution of peripherally derived GH to circulating levels has been proposed to be very low compared to anterior pituitary derived GH, this may suggest that peripheral tissues have a role in terms of regulating the GH levels during late embryogenesis. Papotti et al., (2000) found GHS binding sites in a

variety of human tissues including skeletal muscles. Increased expression of the GHSR during the late stages of embryonic development indicate a potential role of the GHS during the late pre-hatch development of the chick, an effect that may be independent of GH. The ability of GHS's to induce GH secretion from the chicken (Bowers et al., 1984, Geris et al., 2001) and identification of the endogenous ligand, ghrelin, for the GHSR have shown another potential GH regulatory mechanism (Kojima et al., 1999). Thus, it would be of interest to know what affect IGF-I administration has on the synthesis and/or secretion of endogenous GHS's, such as ghrelin.

The expression pattern of the GHSR gene during embryonic development indicates an intricate role in the regulation of peripheral GH secretion during chicken embryonic development. The increased expression on E7 following rhIGF-I administration indicates that the increased posthatch muscle growth observed by Kocamis et al., (1998) may have resulted from increased GH release originating from the peripheral tissues during early embryonic development. As a future study, a real time PCR approach should be used to measure the precise amount of mRNAs for GHSR gene in response to *in ovo* administration of rhIGF-I. The data presented here indicate a critical role for the GHSR and potentially its endogenous ligand in chicken embryonic development.

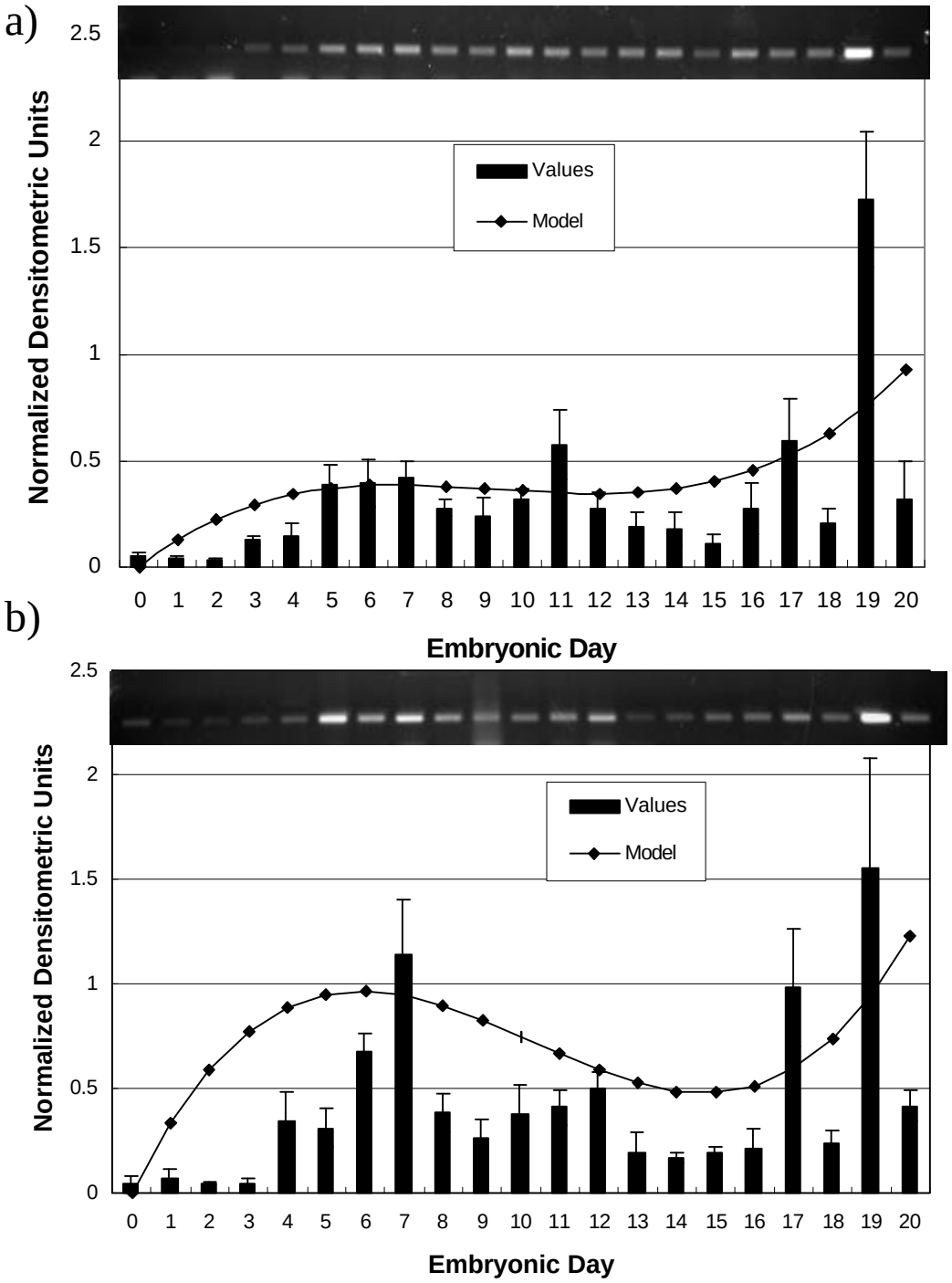


Figure 2-1 - Steady-state levels of control (a) and rhIGF-I treated (b) group GHSR mRNAs in whole embryo (E 0 to E 6, n= 6 per day), cranial halves (E 7 and E 8, n= 6 per day), and pectoralis muscle (E 9 to E 20, n= 4 per day) during chicken embryonic development. The bands for GHSR mRNA were analyzed by densitometry and the integration values (mean ± SD), after normalization to 18s rRNA, expressed as normalized densitometric units at each sampling day.

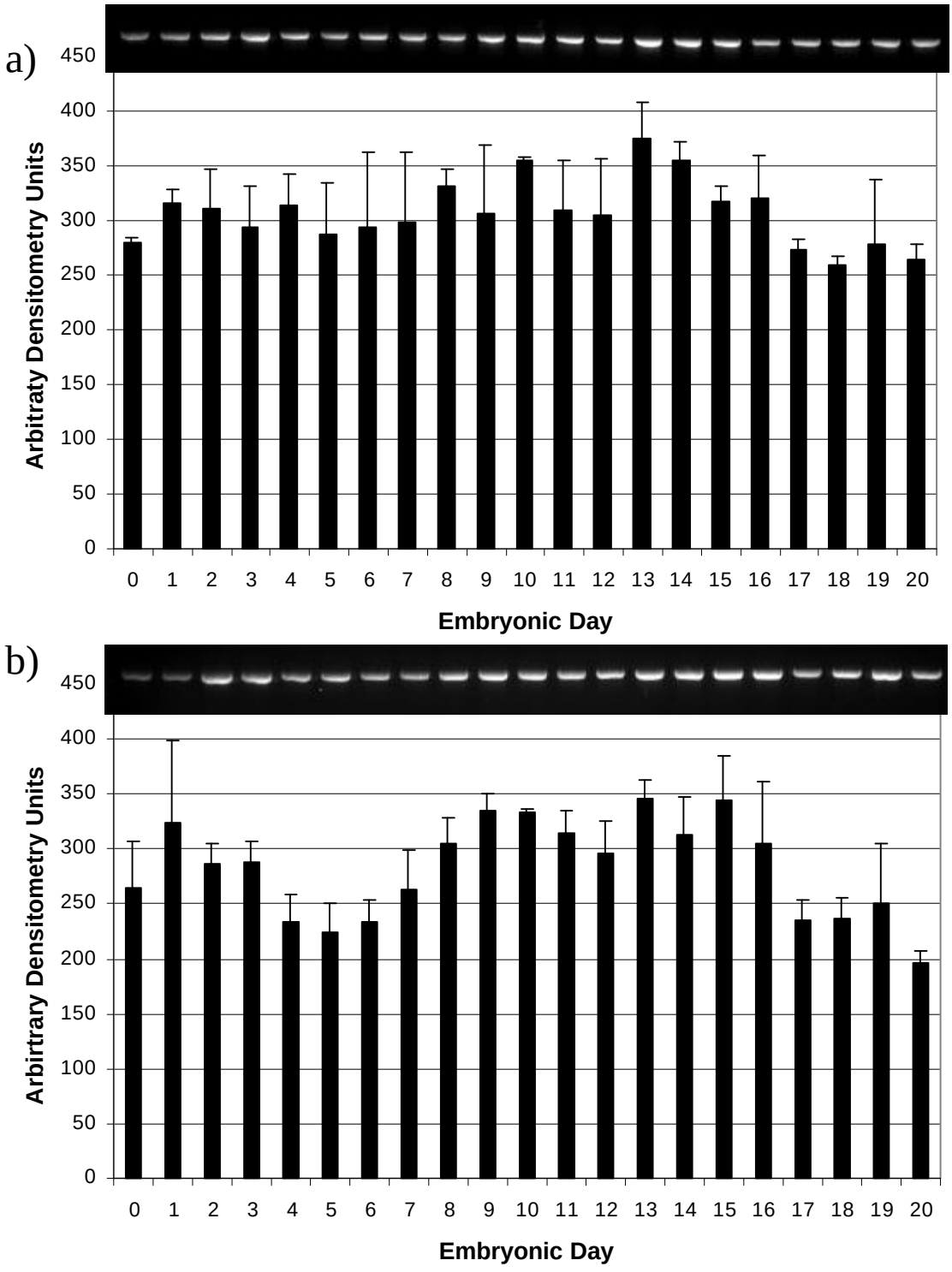


Figure 2-2 - Steady-state levels of control (a) and rhIGF-I treated (b) group 18s rRNA mRNAs in whole embryo (E 0 to E 6, n= 6 per day), cranial halves (E 7 and E 8, n= 6 per day), and pectoralis muscle (E 9 to E 20, n= 4 per day) during chicken embryonic development. The 18 rRNA bands were analyzed by densitometry and the arbitrary densitometric units at each sampling day.

Chapter 3

Expression of Ghrelin, the Endogenous Growth Hormone Secretagogue in the Myostatin Knockout Mouse

INTRODUCTION

Ghrelin, the recently identified endogenous ligand for the growth hormone secretagogue receptor (GHSR), has been demonstrated *in vitro* (Kojima et al., 1999, Hosoda et al., 2000) and *in vivo* both in rats (Date et al., 2000) and humans (Takaya et al., 2000) to increase growth hormone (GH) release. In addition to the GH releasing role, several GH-independent actions of ghrelin have been demonstrated. Tschöp et al. (2000) found that a single injection of ghrelin daily resulted in an increased respiratory quotient indicating increased utilization of carbohydrate and sparing of fat, reflected in the increased fat mass of the treated animals. Additionally, ghrelin was shown to significantly increase feed intake in humans (Wren et al., 2001b) and rodents (Wren et al., 2001a). Stimulation of neuropeptide Y (NPY) and agouti related peptide (AGRP) expression in the arcuate nucleus of the hypothalamus has been proposed as the regulatory mechanism of feed intake (Kamegai et al., 2001; Lawrence et al., 2002). The combination of induced feed intake and increased fat deposition has lead to significant interest in the relationship between ghrelin and human obesisty.

Myostatin, also known as growth differentiation factor-8, a recently identified member of the TGF-β family, has been demonstrated to be a negative regulator of muscle growth (McPheron et al., 1997). In addition to muscle growth, recent studies have indicated that loss of myostatin function reduced adipogensis. Attenuation of myostatin activity through gene knockout (Lin et al., 2002) or expression of the myostatin prodomain (Yang et al., 2001) resulted in a significant reduction in the accumulation of fat during the growing period. Additionally, the significant reductions of fat mass in crosses of myostatin knockout mice with Agouti lethal (A^y) or the Obese (*ob/ob*)

genotype suggesting an indirect role of myostatin on fat deposition, acting to alter glucose metabolism (McPherron and Lee, 2002). Cell specific activity of myostatin has been demonstrated by alteration of adipocyte specific gene expression in 3T3-L1 preadipocytes cultures treated with myostatin (Kim et al., 2001).

In the current study, we have assessed changes in the expression of ghrelin in several tissues of the myostatin knockout mouse. Based on the reduced fat deposition in the myostatin knockout mouse, we expect that the loss of myostatin may down regulated expression of ghrelin and thereby limit adipogensis in these mice.

MATERIAL AND METHODS

Animals

Nine-month-old male myostatin knockout (n= 3, SVJ/129) and control mice (n= 3, C57Bl/6) 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 from myostatin knockout and control mouse brain, heart, liver, kidney, and skeletal muscle (pectoralis) tissues using the Tri-Reagent (Molecular Research Center Inc., Cincinnati, OH) 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 (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 -80ºC.

Reverse transcription (RT) was performed using the murine maloney leukemia virus reverse transcriptase (Promega, Madison, WI) as previously described (Kocamis et al., 2002). For the PCR reaction, 2 µl of RT reaction mixture were added to 50 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_2O. For β-Actin, the PCR reaction started with one cycle consisting of 94^o C for 5 minutes, an annealing step 55^o C for 1 minute and extension at 72^o C for 1 minute. The first cycle was followed by 25 cycles consisting of 1 min intervals of 94^o C, followed by 55^o C, followed by 72^o C. The reaction was concluded with a 10-min extension step at 72°C. For ghrelin, touchdown PCR was run. This program consisted of a 5-minute denaturation step at 94^o C, followed by 5 cycles in which the initial annealing temperature of 67^o C was reduced by 1^o C per cycle, then 30 cycles in which the annealing temperature was 63^o C. 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 (volume was replaced by water) 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 ghrelin were designed on the basis of published sequences of mouse ghrelin (Kojima et al., 1999). The sequence of the forward primer was 5'-TGAGCCCAGAGCACCAGAAA-3', and the reverse primer was 5'-

GGCCTGTCCGTGGTTACTT-3'. Forward and reverse primers predicted a PCR product of 285 base pairs (bp), which corresponds to bases (98-382) of the sequence. Forward and reverse primers for β-actin were predicted to amplify a 285 bp product as previously published (Yamamura et al., 1991), that was used as an internal standard to verify the level of amplification. For β-Actin, the sequence of the forward primer was 5'-TCATGAAGTGTGACGTTGACATCCGT-3', and the reverse primer was 5'-CCTAGAAGCATTTGCGGTGCACGATG-3'.

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

Statistical Analysis

Probability of differences of least-square means was used to compare densitometric values (General Linear Model procedure of SAS, SAS Institute Inc., Cary, NC).

RESULTS

β-actin expression was not different between the control and knockout tissues in any of the tissues sampled (Figure 3-1). Ghrelin was detected in all tissues sampled (Figure 3-2) with the highest expression in the kidney, followed by the heart, liver, pectoralis muscle and heart (Figure 3-2). Expression was similar between the control and knockout tissues for brain, liver and kidney (Figure 3-2). Ghrelin expression was significantly reduced in the heart tissue ($P<0.05$, Figure 3-2) and showed a tendency to be reduced in the pectoralis ($P= 0.07$, figure 3-2) in the myostatin knockout as compared to the control.

DISCUSSION

Myostatin, a negative regulator of muscle growth, has been recently found to effect the distribution of fat in the growing mouse (Lin et al., 2002). Therefore, the current study compared the expression of ghrelin, an adipogenic factor, in the tissues of the myostatin knockout and wild type mouse. We found expression of ghrelin in all tissues examined with highest expression in the kidney, with lower expression in the brain, liver, pectoralis, and heart (Figure 3-2). Gnanapavan et al. (2002), using real-time PCR, demonstrated a similar transcript distribution for ghrelin in the human tissues.

Attenuation of myostatin activity via gene knockout (Lin et al., 2002; McPherron and Lee, 2002) or transgenic myostatin pro-domain expression (Yang et al., 2001) caused a significant reduction in fat mass. In the current study, we found ghrelin expression to be significantly reduced in the heart ($P < 0.05$) and tended to be reduced in the pectoralis muscle ($P = 0.07$, figure 3-2) in the myostatin knockout mouse. Previous studies have found myostatin expression in muscle tissue (McPherron and Lee, 1997) and in the Purkinje fibers of the heart (Sharma et al., 1999). Taken together, this may indicate that myostatin, acting in an autocrine/paracrine manner, regulates the expression of ghrelin. The loss of myostatin had no effect on the expression of ghrelin in the brain, kidney and liver (figure 3-2). To date, no studies have found the expression of myostatin in these tissues. Tissue colocalization and effects of gene knockout/transgenics further support the local effects of myostatin on ghrelin gene expression.

The loss of myostatin activity in two genetic models of obesity, the A^y and ob/ob mice resulted in a partial reduction in fat accumulation (McPherron and Lee, 2002). In

each case, the down regulation of ghrelin in the myostatin expressing tissues are consistent with the observed changes in fat accumulation. In the A^y mouse, expression of the agouti peptide in a variety of peripheral tissues has been proposed as causative agent for the increased fat deposition (reviewed by Michaud et al., 1997). The increased feed intake and fat accumulation as a result of ghrelin administration has been proposed to be the result of increased agouti-related protein expression in the arcuate nucleus of the hypothalamus (Kamegai et al., 2000). The reduction in ghrelin expression in the peripheral tissues may attenuate ghrelin's induction of agouti protein in the surrounding tissues and thereby attenuate fat deposition. In the *ob/ob* mouse, the development of obesity is the result of a mutation in the leptin gene, resulting in a truncated protein, altering both the central and adipocyte specific effects of leptin on fat accumulation (Huang and Li, 2000). In rats, intracentraventricular administration of ghrelin has been demonstrated to antagonize leptin activity in the hypothalamus (Shintani et al., 2001). The reduction of ghrelin expression with the loss of myostatin activity may act to remove a positive signal on the adipocyte to increase adipose tissue growth and development. Therefore, the local changes in the ghrelin expression, as a result of loss of myostatin activity, may describe one mechanism by which myostatin regulates fat deposition.

Although circulating levels of ghrelin were not measured in the current study, the lack of changes in ghrelin expression in the tissues that do not express myostatin indicates that the changes in tissue-level ghrelin expression was not altered with the loss of myostatin activity. A lack of systemic reduction in ghrelin levels may explain the only partial suppression of fat accumulation observed in the myostatin knockout mouse (McPherron and Lee, 2002). That is, maintenance of circulating ghrelin concentrations,

i.e. gastric production, may act to allow accumulation of some fat in the myostatin knockout mouse. The role of ghrelin in fat accumulation and growth of the myostatin knockout mouse may allow a better understanding of the underlying mechanism by which myostatin increases muscle mass and reduces fat accumulation.

The current study attempted to use the expression of ghrelin in the myostatin knockout tissue to elucidate a potential mechanism by which fat accumulation is attenuated in the myostatin knockout mouse. However, the exact mechanism regulating this expression has yet to be fully elucidated. McPherron and Lee (2002) suggest that myostatin may act either directly on the adipose tissue or indirectly by altering hormone secretion in the muscle to regulate the fat accumulation. The current study would support the indirect manner of myostatin regulating fat deposition. That is, loss of myostatin acting at the level of the muscle and heart may down regulate the secretion of ghrelin and thereby reduce the accumulation of fat. Further understanding of the relationship between myostatin and ghrelin expression may provide insight into fat deposition, having implications in both the medical and agricultural fields.

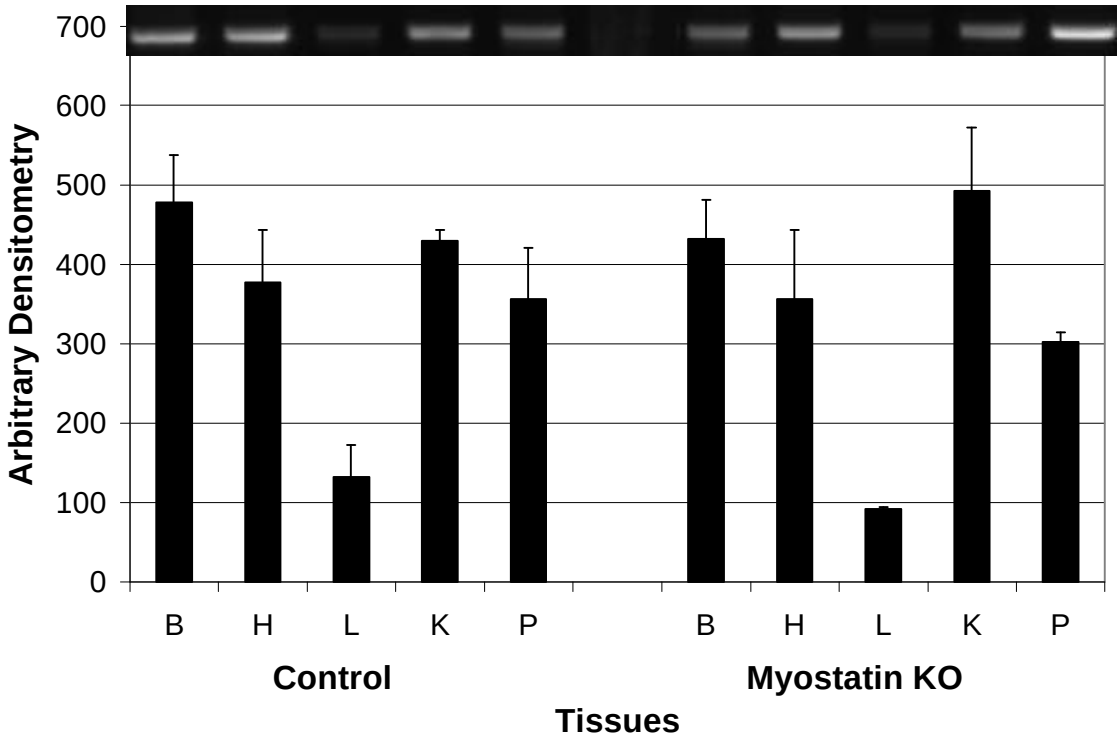


Figure 3-1- Steady-state levels of β-actin mRNA in brain (B), heart (H), liver (L), kidney (K), pectoralis (P) muscle of control (n= 3) and myostatin knockout mice (n= 3) tissues. The bands for β-actin were analyzed by densitometry and the integration values (mean ± SD) were expressed in arbitrary units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

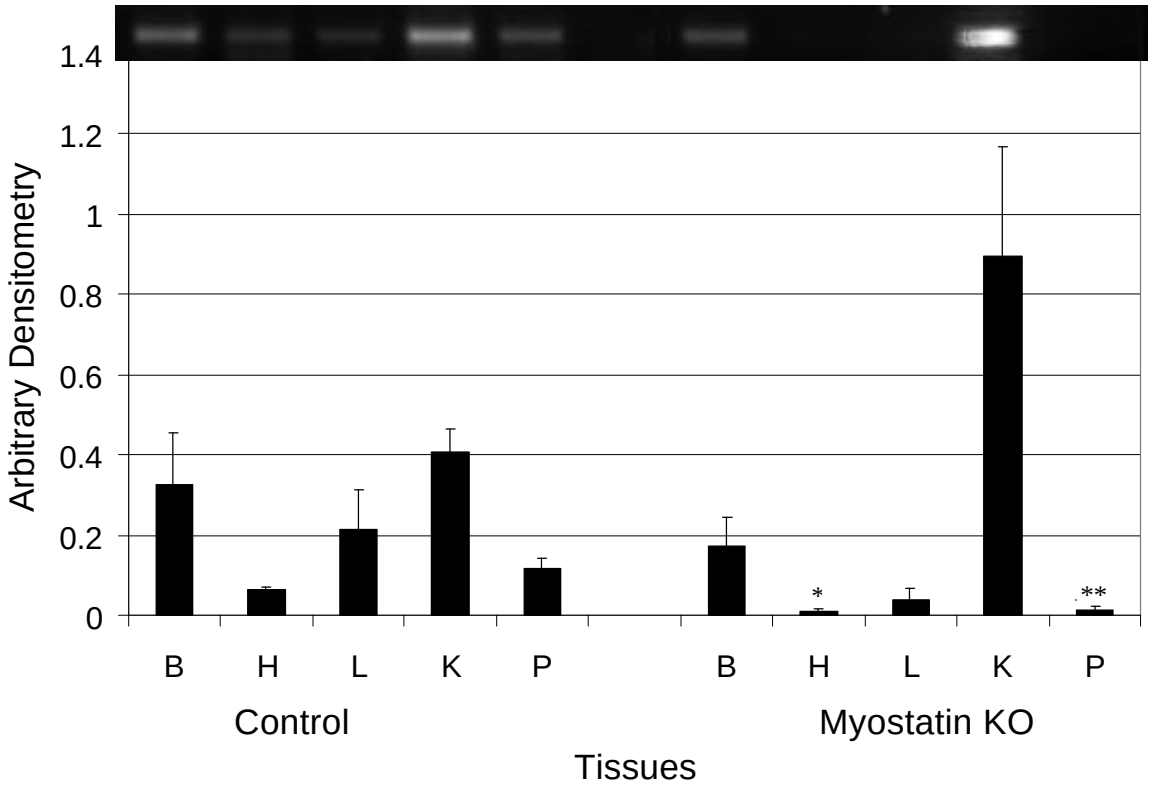


Figure 3-2 - Steady-state levels of ghrelin mRNA in brain (B), heart (H), liver (L), kidney (K), and pectoralis (P) muscle of control (n= 3) and myostatin knockout mice (n= 3) tissues. The bands for ghrelin were analyzed by densitometry and the integration values (mean ± SD), after normalization to β actin, were expressed in arbitrary units for each tissue (*-P<0.05, **-P=0.07 compared to the Control Tissue).

CONCLUSIONS AND FUTURE STUDIES

The growth and development of muscle is regulated by a well-orchestrated interaction between positive and negative stimuli resulting in the proper distribution of the functional unit of support and locomotion. Gaining a better understanding of the mechanisms regulating muscle growth and development will be useful to both the improvement of animal production and treatment of human disease. In the work presented here, we have tried to determine the relationship between a potential enhancer of muscle growth, ghrelin, and a negative regulator of muscle growth, myostatin, in the broiler chicken. Although the primary focus of this work was to improve muscle growth in the meat type chicken, the implications of the work completed here may potentially have implications well beyond improved broiler production.

In mammalian species, attenuation of active myostatin has been shown to greatly increase muscle mass. In the chicken, the lack of an identifiable double muscled phenotype and difficulties with gene knockout applications have limited the ability to study attenuation of myostatin activity in the chicken. Taking advantage of the binding of the prodomain of myostatin to the mature protein (Figure I2) to preclude receptor binding has recently been found to significantly increase muscle mass of the mouse (Yang et al., 2001). We have used *in ovo* administration of the prodomain of myostatin during secondary muscle fiber proliferation to significantly increase muscle mass of female chickens at six weeks of age. The significance of this data is twofold. First, this indicates a similar role for myostatin in pre-natal muscle growth and development in the chicken as has been observed in the mouse (McPherron et al., 1997). However, the increased effect found in the female birds may indicate a sexually dimorphic manner of

action in the chicken. Additionally, with the high proportion of broiler producers (~80%) currently using *in ovo* vaccination, this method could provide a method that is easily adaptable to the broiler industry to reduce the discrepancy between male and female broilers during production greatly increasing the productivity of the individual houses.

In contrast to the negative regulation of myostatin on muscle growth, GH has been found to significantly increase muscle growth and development in mammalian species (reviewed by Etherton and Bouman, 1998). Although similar results have not been consistently demonstrated in the chicken, the absolute requirement of GH for normal growth indicates a significant role in broiler growth and development (reviewed by Scanes et al., 1986). Recently, Harvey et al., (2000) have found GH immunoreactivity in a variety of cells of the early chick embryo indicating a role for GH during early embryonic development in the chicken. However, they have not investigated the mechanism regulating the secretion of GH during this timing of embryonic development. The unresponsiveness of the chicken embryos to the hypothalamic secretory proteins, GHRH and TRH, before embryonic day 14, indicates an independent mechanism regulating this GH secretion (Darras et al., 1994). Identification of a cDNA corresponding to the GHSR (Toogood et al., 1999) and GHS ability to increase GH secretion both directly in the anterior pituitary and through TRH in the hypothalamus (Geris et al., 2001) indicate the existence of a GHS mechanism in the chicken. This leads to the possibility that the GHSR is acting to regulate the expression of GH in the peripheral tissues during embryonic development. We have found increased expression of the GHSR beginning on E5, which corresponds positively with the observed increase

in GH immunoreactivity between E3 and E6 found by Harvey et al., (2000). Therefore, we would suggest that the GHSR is acting to increase expression of GH during early embryonic development. Although the endogenous ligand for the GHSR has yet to be identified in the chicken (see below), the overlapping expression of the GHSR and GH suggests that GHS administration during this time period would significantly increase the release of GH in the early embryo, and thereby increase postnatal muscle growth and therefore broiler productivity. Additionally, the increase in GHSR expression following rhIGF-I administration on E3 may indicate a significant role for GH in the observed increase in muscle growth (Kocamis et al., 1998).

As alluded to above, the role for the GHS in the secretion of GH during chicken embryonic development indicates a significant role for the receptors endogenous ligand, ghrelin, in growth and development of the chicken. However, attempts in this lab to identify this ligand have proven futile. We have identified two changes in the approaches used that could prove useful for the identification of this peptide. We have used PCR to generate a partial sequence to be used as a probe in cDNA library screening to isolate the cDNA representing chicken ghrelin. However, 3 sets of primers designed to the mouse sequence and a set of primers based on chicken motilin (see identification of ghrelin as motilin-related peptide above) were unable to generate a fragment showing homology to any identified ghrelin sequences. Additionally, utilization of a 282-bp fragment of mouse cDNA with 100% homology to mouse des-Gln^{14}-ghrelin was unable to identify a ghrelin clone with screening proventriculus, heart and adipose cDNA libraries and in southern blot analysis of chicken genomic DNA. This indicated a significant divergence between the mouse and chicken ghrelin genes. This conclusion is supported by the lack of

significant homology between specices; for example there is only 29 % amino acid homology between mouse and bullfrog ghrelin cDNA (Figure C1; Kiaya et al., 2001). The common feature to all of the identified ghrelin sequences is the octanoylation of amino acid 3, serine in the mammal species and threonine in the bullfrog. This modification has been shown to be an absolute requirement for receptor binding (Bernarek et al., 2000). Therefore, a reverse pharmacologial approach could be used to identify the chicken ghrelin protein. That is, using a cell line transfected with the full length mouse GHSR, or following isolation of the chicken full length sequence, one could screen several chicken tissues to purify the proteins with the ability to bind to and stimulate the GHSR in the chicken. Because of the divergence in the ghrelin sequences, what we are suggesting is rather than utilizing nucleic acid techniques, one could use a technique similar to the method used by Kojima et al. (1999), described above, to identify ghrelin in the chicken.

Although we do not want to understate the difficulty in the establishment of the cell lines, this process would allow screening of a variety of tissues in the chicken without requiring extensive processing, i.e. construction of the cDNA library. This process could eliminate the second complication that may have limited our ability to identify this ligand in the chicken. We had used mammalian data to postulate that expression of ghrelin would be greatest in the proventriculus, i.e. the glandular region of the chicken stomach. Using a pool of mRNA from the proventriculus at weeks 1 to 5, the rapid growing phase of the chicken's life, we constructed a cDNA library and screened it for the ghrelin cDNA sequence. This library was found to have an accepible titer, 3.1 x 10^9 pfu/mL, and contained relatively large inserts, average size at about 900 bp.

Additionally, the successful analysis of 134 ESTs has identified cDNA of proteins specific to the stomach (see the appendix). However, part of the inability to identify ghrelin may have stemmed from looking in the incorrect tissue. A recent study has found no ghrelin immunoreactivity in the mucosal cells of the proventriculus, rather a rat antibody showed binding only in the hypothalamus (Ahmed and Harvey, 2002). This would suggest future attempts to characterize ghrelin at the nucleic acid level must include analysis of this region of the brain.

Finally, we attempted to determine if a relationship between myostatin and ghrelin exists in the determination of muscle and fat mass of the mouse. In this study we used RT-PCR to determine the expression of ghrelin in several tissues of the myostatin knockout mouse. We found a reduction in ghrelin expression in the heart and pectoralis muscle of the myostatin knockout mouse. Although these data represent a preliminary assessment of the alteration in ghrelin expression, we have not looked at the active protein levels in the serum. They may indicate an interaction between ghrelin and myostatin in the partitioning of the body tissues during growth and development. That is, loss of myostatin activity in the mouse (myostatin knockout mouse; McPherron and Lee, 2002) and in double muscled cattle (Bass et al., 1999) leads to a much leaner animal with a significant increase in muscle mass. The increased muscle mass is proposed to result from the loss of anti-proliferative effects of myostatin on the muscle cells (reviewed by Sharma et al., 2001). However, the identification of the myostatin mutation in cattle that do not show double muscling indicated the presence of additional factors involved in the development of this phenotype (Smith et al., 2000). The data collected here may provide evidence for the involvement of ghrelin as one of these factors. For example, in the

South Devon breed of cattle, not considered to be double muscled, the myostatin gene shows the same mutation identified in the Belgian Blue breed, the poster child of double muscling. We may speculate that ghrelin expression is not attenuated in the muscle of the South Devon breed resulting in either direct or indirect effects on muscle growth. Ghrelin may act directly on the muscle to cause reduced proliferation and thereby mediate the loss of the double muscled phenotype. Alternatively, ghrelin may cause a shift in the metabolism of the cells during muscle growth. That is, ghrelin may act on the fat cells to increase energy and nutrient uptake by the fat, causing a shift from lean to fat tissue deposition and thereby not allowing for as significant increase in the muscle mass.

In either case, further research remains to be conducted. The direct effects of ghrelin could be determined by treatment of muscle cells in culture with exougenously-supplied ghrelin. The cells could not only be monitored for changes in proliferation, but also for nutrient utilization. However, the indirect effects would be significantly more difficult to assess. One could choose to use a co-culture system with fat and muscle cells incubated together. Although, this would allow assessment of the interactive effects of ghrelin, however, it would remove the systemic effects from the equation. Therefore, a more complete picture of the effects could be gathered by measuring the effects of ghrelin administration on muscle during growth and developmentOur data suggests a reduction in ghrelin expression in the mouse muscle following loss of myostatin activity. The mechanism resulting in the reduction of ghrelin expresion may involve more factors beyond the limited scope of the current study. Although much work has been done in the mouse with attenuation of myostatin activity, to date no complete hormone profile has been published. The observed decrease in the fat deposition concurrent with increased

muscle mass of the myostatin knockout mouse indicates that a variety of changes in the hormone profile of the animal have occurred, which have yet to be fully elucidated.

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SIGNAL PEPTIDE
Bullfrog      1  MNFGKAAIFGVVL-FCLLWTEGAQA  24
Human         1  MPSPGTVC-SLLLLGML-WLDLAMA  23
Rat           1  MVSSATIC-SLLLLSML-WMDMAMA  23
Mouse         1  MLSSGTIC-SLLLLSML-WMDMAMA  23
Bovin         1  MPAPWTIC-SLLLLSVL-CMDLAMA  23
Pig           1  MPSTGTIC-SLLLLSVLLMADLAMA  24
Dog           1  MPSLGTMC-SLLLFSVLWV-DLAMA  23
                 *          *   *    * *

GHRELIN
Bullfrog     25 GLTFLSPADMQKIAERQSQNKLRHGNMN--  53
Human        24 GSSFLSP-EHQRVQQRKESKK-PPAKLQPR  51
Rat          24 GSSFLSP-EHQKAQQRKESKK-PPAKLQPR  51
Mouse        24 GSSFLSP-EHQKAQQRKESKK-PPAKLQPR  51
Bovin        24 GSSFLSP-EHQKL-QRKEAKK-PSGRLKPR  50
Pig          25 GSSFLSP-EHQKVQQRKESKK-PAAKLKPR  52
Dog          24 GSSFLSP-EHQKLQQRKESKK-PPAKLQPR  51
                * ****   *    *    *

C-TERMINAL PEPTIDE
Bullfrog     54 RRGVEDDLAGEEIGVTFPLDMKMTQEQFQ-KQRAAVQ--DF-LYSSLLSLGS-VQDT--EDKNENPQSQ  114
Human        52 --ALAGWLRPEDGGQAEGAEDEL-EVRFNAPFDVGIKLSGVQYQQHSQALGKFLQDILWEEAKEAPADK  117
Rat          52 --ALEGWLHPEDRGQAEEAEEEL-EIRFNAPFDVGIKLSGAQYQQHGRALGKFLQDILWEEVKEAPANK  117
Mouse        52 --ALEGWLHPEDRGQAEETEEEL-EIRFNAPFDVGIKLSGAQYQQHGRALGKFLQDILWEEVKEAPADK  117
Bovin        51 --TLEGQFDPEVGSQAEGAEDEL-EIRFNAPFNIGIKLAGAQSLQHGQTLGKFLQDILWEEAEETLANE  116
Pig          53 --ALEGWLGPEDSGEVEGTEDKL-EIRFNAPCDVGIKLSGAQSDQHGQPLGKFLQDILWEEVTEAPADK  118
Dog          52 --ALEGSLGPEDTSQVEEAEDEL-EIRFNAPFDVGIKLSGPQYHQHGQALGKFLQEVLWEDTNEALADE  117
                            *                   *                      **   *    *   *
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Figure C1- Aligment of Bullfrog Ghrelin amino acid sequence with the identified Mammal Ghrelin Sequences.

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Appendix 1

Chicken Proventriculus EST Clone Identities

Total 134 EST Sequences

117 Uniques Sequences Representing 63 Identified Genes

64 Unknown Sequences

Clone ID	Gene Identity	Score	Expected	Homology	% Homology
1-47	60S Ribosomal Protein L34 (Mouse)	291	3.00E-76	296/345	85%
1-27	Chicken CaM gene encoding calmodulin	345	1.00E-92	221/242	91%
1-28	Chicken mRNA for acidic ribosomal protein P1	494	1.00E-137	259/261	99%
2-87	Chicken mRNA for ribosomal protein L5	716	0	431/452	95%
1-78	Chicken mRNA for ribosomal protein L5	755	0	429/440	97%
1-16	Chicken neuropeptide Y	75.8	3.00E-11	38/38	100%
1-66	Felis catus cell-type fibroblast mutant beta-glucuronidase (GUSB)	52	5.00E-04	43/49	87%
1-52	G.domesticus mRNA BTG1	753	0	420/427	98%
2-33	Gallus domesticus helix-loop-helix transcription factor sequence	565	1.00E-158	348/365	95%
1-77	Gallus gallus beta-actin mRNA	779	0	443/452	98%
2-44	Gallus gallus calmodulin mRNA	597	1.00E-168	376/397	94%
1-43	Gallus gallus clone CW nonLTR retrotransposon CR1	75.8	2.00E-11	61/69	88%
2-59	Gallus gallus cPgA mRNA for pepsinogen A	545	1.00E-152	364/393	92%
1-09	Gallus gallus cPgA mRNA for pepsinogen A	545	1.00E-152	343/361	95%
1-65	Gallus gallus cPgA mRNA for pepsinogen A	682	0	417/437	95%
2-50	Gallus gallus cPgA mRNA for pepsinogen A	607	1.00E-171	371/390	95%
1-25	Gallus gallus cPgA mRNA for pepsinogen A	696	0	414/427	96%
1-70	Gallus gallus cPgA mRNA for pepsinogen A	555	1.00E-156	308/316	97%
1-94	Gallus gallus cPgA mRNA for pepsinogen A	761	0	438/447	97%
2-45	Gallus gallus cPgA mRNA for pepsinogen A	626	1.00E-177	338/347	97%

Clone ID	Gene Identity	Score	Expected	Homology	% Homology
2-71	Gallus gallus cPgC mRNA for pepsinogen C	615	1.00E-173	373/390	95%
2-70	Gallus gallus domesticus H+ K+ ATPase beta subunit mRNA	509	1.00E-142	346/372	93%
2-48	Gallus gallus kinase related protein gene	656	0	373/391	95%
2-69	Gallus gallus kinase related protein gene	678	0	391/407	96%
1-60	Gallus gallus kinase related protein mRNA	743	0	435/451	96%
1-19	Gallus gallus kinesin light chain mRNA	234	5.00E-59	125/126	99%
2-37	Gallus gallus mitochondrial genome	539	1.00E-151	299/306	97%
1-76	Gallus gallus mitochondrial genome	787	0	407/409	99%
2-10	Gallus gallus mitochondrial genome	583	1.00E-164	304/306	99%
1-41	Gallus gallus mitochondrion, complete genome	317	3.00E-84	187/194	96%
1-68	Gallus gallus mRNA for human bbc1 (breast basic conserved gene) product homologue	660	0	388/401	96%
1-12	Gallus gallus mRNA for matrix GLA protein	700	0	374/377	99%
2-38	Gallus gallus NADH dehydrogenase mRNA	640	0	358/368	97%
1-92	Gallus gallus otokeratin mRNA	817	0	450/456	98%
1-20	Gallus gallus p95 paxillin-kinase linker mRNA	414	1.00E-113	242/248	97%
1-13	Gallus gallus peptide elongation factor 1-beta	317	4.00E-84	183/187	97%
1-89	H.sapiens (xs81)	95.6	4.00E-17	78/87	89%
1-75	Homo sapiens C9orf10 protein	345	2.00E-92	283/315	89%
1-03	Homo sapiens eosinophil chemotactic cytokine	105	4.00E-20	140/169	82%
1-22	Homo sapiens eosinophil chemotactic cytokine	83.8	1.00E-13	81/94	86%
1-50	Homo sapiens eosinophil chemotactic cytokine (TSA1902)	83.8	1.00E-13	99/119	83%
1-72	Homo sapiens eosinophil chemotactic cytokine (TSA1902)	99.6	2.00E-18	132/159	83%
1-87	Homo sapiens eosinophil chemotactic cytokine (TSA1902)	52	5.00E-04	53/62	85%
1-51	Homo sapiens GAJ protein (GAJ)	73.8	9.00E-11	109/132	82%

Clone ID	Gene Identity	Score	Expected	Homology	% Homology
1-71	Homo sapiens geranylgeranyl pyrophosphate synthetase	194	5.00E-47	327/403	81%
2-11	Homo sapiens glutamyl-prolyl-tRNA synthetase (EPRS)	198	4.00E-48	223/264	84%
2-01	Homo sapiens hypothetical protein DKFZP586F1318	107	1.00E-20	129/154	83%
1-30	Homo sapiens insulin-like growth factor binding protein 7	89.7	2.00E-15	136/165	82%
2-43	Homo sapiens KIAA0970 protein (KIAA0970)	151	6.00E-34	272/338	80%
2-67	Homo sapiens profilin 2 (PFN2), transcript variant 1	327	7.00E-87	259/287	90%
2-75	Homo sapiens protein disulfide isomerase related protein (calcium-binding protein, intestinal-related) (ERP70)	67.9	9.00E-09	61/70	87%
2-26	Homo sapiens protein tyrosine phosphatase type IVA, member 1 (PTP4A1)	172	2.00E-40	212/255	83%
1-57	Homo sapiens RAB2, member RAS oncogene family (RAB2)	218	4.00E-54	119/122	97%
1-14	Homo sapiens ribosomal protein L32 (RPL32)	283	7.00E-74	305/359	84%
1-05	Homo sapiens ribosomal protein L9 (RPL9)	250	1.00E-63	285/338	84%
1-26	Homo sapiens signal peptidase 12kDa	141	5.00E-31	161/191	84%
2-25	Homo sapiens similar to DKFZP586O0120 protein	151	6.00E-34	110/122	90%
2-24	Homo sapiens similar to dynein, cytoplasmic, light polypeptide	289	1.00E-75	254/290	87%
1-69	Homo sapiens similar to voltage-dependent anion channel 1	351	4.00E-94	369/431	85%
1-04	Homo sapiens spermine synthase (SMS)	260	1.00E-66	312/371	84%
1-48	Homo sapiens stromal cell derived factor receptor 1 (SDFR1)	73.8	1.00E-10	59/65	90%
1-54	Homo sapiens, ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit	125	4.00E-26	177/215	82%
2-29	Mus musculus KDEL containing protein 1 (Kdel1),	117	1.00E-23	127/150	84%
1-08	Mus musculus nuclear receptor subfamily 2, group F, member 2 (Nr2f2)	46.1	0.017	38/42	90%
1-85	Mus musculus ribosomal protein L27a (Rpl27a)	200	9.00E-49	355/436	81%
1-17	NAD-Dependent Methanol Dehydrogenase (Human)	202	2.00E-49	275/330	83%

Clone ID	Gene Identity	Score	Expected	Homology	% Homology
1-91	Rattus norvegicus ribosomal protein L35a (Rpl35)	202	2.00E-49	252/301	83%
1-02	Ribosomal protein L27a (Mouse)	172	2.00E-40	201/239	84%
1-62	Ribosomal Protein L38 (Mouse)	248	3.00E-63	192/213	90%
1-01, 1-07, 1-10, 1-11, 1-15, 1-23, 1-29, 1-31, 1-32, 1-33, 1-34, 1-35, 1-36, 1-37, 1-38, 1-39, 1-40, 1-42, 1-45, 1-49, 1-53, 1-55, 1-56, 1-63, 1-64, 1-67, 1-74, 1-79, 1-80, 1-81, 1-82, 1-90, 1-93, 1-95, 1-96, 2-08, 2-27, 2-32, 2-34, 2-35, 2-39, 2-40, 2-41, 2-42, 2-47, 2-52, 2-53, 2-54, 2-55, 2-56, 2-57, 2-58, 2-64, 2-65, 2-66, 2-72, 2-73, 2-74, 2-76, 2-79, 2-80, 2-83, 2-90, 2-91	Unknown				

Appendix 2

Uncoupling Protein 3 in the Rainbow Trout (*Oncorhynchus mykiss*): Sequence, Variants and Association with the AvaIII SINE element.

ABSTRACT

A Rainbow Trout Uncoupling Protein 3 (UCP3) cDNA clone, encoding a 309 amino acid protein, was cloned and sequenced from a liver cDNA library. Two different splice variants designated UCP3-v1 and UCP3-v2, were found through liver cDNA library screening using Rainbow Trout UPC3 cDNA clone as probe. UCP3-v1 has 3 insertions in the UCP3 cDNA: the first insertion (133 bp), the second insertion (141 bp), and the third insertion (370 bp) were located 126 bp, 334 bp, and 532 bp downstream from start codon respectively. UCP3-v2 contained a single insertion, identical in sequence and location to the second insertion of UCP3-v1. UPC3, a mitochondrial protein, functions to modulate the efficiency of oxidative phosphorylation. Human and rodent UCP3s are highly expressed in skeletal muscle and brown adipose tissue. In contrast to mammalian studies, RT-PCR and Southern Blot Analyses of the Rainbow Trout demonstrated that UCP3 was not restricted to skeletal muscle and adipose tissue. Expression was detected in all tissues examined with the highest levels found in liver and heart. UCP3, UCP3-v1, and UCP3-v2 all contain an Ava III Short Interspersed Element (SINE), located in the 3'-UTR. PCR using primers from the Ava III SINE and the UCP3 3'-UTR region indicate that the UCP3 cDNA is structurally conserved among Salmonids and that these primers may be useful for Salmonid species genotyping.

INTRODUCTION

Short interspersed repetitive elements (SINEs) are non-viral retrotransposons incorporated into the genome of a wide variety of organisms (Singer, 1982). SINEs are commonly referred to as tRNA pseudogenes as a result of their homology to tRNA sequences (Okada, 1991). In salmonid fish, three classes of SINE elements have been discovered: SmaI family, HpaI family and FokI family. An additional group, the HpaI-related AvaIII family, is common to many salmonid species (Kido et al., 1994). SINE elements, by sequence and quantity, are used as phylogenetic markers (Murata et al., 1996), and the identification of a common SINE element in the transcribed portion of a particular gene may indicate the evolutionary conservation of the gene.

Over the past 30 years, there has been extensive research into the mechanism of non-shivering thermogenesis and the importance of brown adipose tissue (BAT) in the temperature regulation of infants (for review see Nicholls, 2001). This work led to the identification of uncoupling protein 1 (UCP1) in the BAT, a mitochondrial carrier protein with the ability to increase heat production by altering the proton permeability of the inner mitochondrial membrane (for review see Villarroya, et al., 2001). Subsequently, two other members of the UCP family have been identified, UCP2 and UCP3. UCP2, classified according to the aa sequence homology to UCP1, is expressed in a variety of tissues and is thought to serve a similar function to UCP1, but its true function is yet to be fully elucidated (for review see Ricquier and Bouillaud, 2000). An additional UCP (UCP3),identified by the 57% aa homology to UCP1, was found to be primarily expressed in skeletal muscle with lower expression in BAT and cardiac muscle. (Fleury et al., 1997). In addition to the proton shunting ability, recent studies indicate a

significantly different role for UCP3 in the regulation of fatty acid metabolism. During periods of starvation, i.e. a shift in the muscle to primarily lipid catabolism, UCP3 expression is upregulated in the muscle, with the fast glycolytic muscle showing the greatest response (Hildebrant and Neufer, 2000). In the mouse, the connection between UCP3 and fatty acid metabolism is further reflected in the mapping of markers of obesity and hyperinsulemia in close proximity to the UCP3 gene locus on chromosome 7 (Fleury et al., 1997).

Two alternatively spliced forms of the UCP3 gene have been identified in human skeletal muscle. The long transcript ($UCP3_L$) includes a small potion of the 6^{th} exon and a 7^{th} exon, while the short transcript ($UCP3_S$) includes only the 1^{st} 6 exons (Solanes et al., 1997). Based on the cDNA sequences of the two variants, human UCP3 is represented by proteins of 312 aa ($UCP3_L$) and 275 aa ($UCP3_S$) found on the inner mitochondrial membrane (Solanes et al., 1997). However, in the mouse and human, research indicates that only the long transcript is translated into an active protein (for review see Harper et al., 2001).

In a preliminary experiment, we sought to determine identity of gene transcripts containing the HpaI-Related AvaIII SINE element in the Rainbow Trout. From this work, we had isolated a partial sequence of the Rainbow Trout UCP3. With the potential for metabolic regulatory roles for UCP3 in the Rainbow Trout, we decided to look specifically at this gene transcript. Therefore the objective of the current work, was to determine the full length UCP3 sequence in Rainbow Trout, tissue distribution, identity of any splice variants and conservation of the AvaIII SINE element found in the 3' UTR of the UCP3 gene in other salmonid species.

MATERIALS AND METHODS

Determination of Identity of Genes Expressing AvaIII SINE

A liver cDNA library, prepared in λ ZapII vectors, were kindly provided by Dr. Joe Brunelli of Washington State University. During routine EST screening of this library, a clone was isolated that contained a putative AvaIII SINE. A single PCR primer was designed using GeneTools 1.0 (BioTools, Inc., Edmonton, AB, Canada), within the SINE element. The primer sequence was 5'-TTAACCCAACCCCTCTGAATCAGAG-3', which was purchased from GIbco BRL (now Invitrogen, Corp., Carlsbad, Ca). PCR screening of the excised cDNA library, using the AvaIII primer and either the T7 or T3 promoter primers (Promega, Madison, WI), was performed as follows. Twenty-four μL of a PCR cocktail containing: 2.5 μL of 10x Taq buffer (1.5 mM $MgCl_2$ final concentration), 0.5 μL dNTPs (final concentration 0.2 mM each), 1 μL of AvaIII primer (4 ng/mL final concentation) and 5 μL T3 or T7 primer (4 ng/mL final concentration), 0.5 μL (2.5 U) of Taq DNA Polymerase (Display Systems Biotech, Vista, CA) and 14.5 μL of nuclease free water was added to 1 μL excised liver cDNA library phagemid. The PCR started with one cycle consisting of denaturing (94°C) for 4 min, annealing (55°C) for 1 min, and extension (72°C) for 1 min. The first cycle was followed by 30 cycles consisting of 1 min intervals of denaturing at 94°C followed by annealing at 55°C followed by extension at 72°C. These cycles were followed by a final 10 min extension step (72°C). The products were separated by gel electrophoresis (1% agarose) and stained with ethidium bromide to determine the sizes of the amplicons (see Figure 1).

The PCR products were subject to TA Cloning (pCR 4 Vector, Invitrogen, Corp., Carlsbad, Ca), and plasmid insert size was determined by EcoRI restriction digestion and gel electrophoresis analysis. Plasmids containing unique amplicons were sequenced using the Big Dye Terminator method (PE Biosystems Co., Foster City, CA) on an automated ABI 310 DNA sequencer (PE Biosystems Co., Foster City, CA). The identity of each insert was determined by using the NCBI Blast search of the Gen Bank Data Base. Plasmid DNA was stored at –20ºC.

Rainbow Trout Liver cDNA Library Screening For UCP3

The UCP3 cDNA was found among the expressed sequences cloned and determined to contain an AvaIII SINE. Because this gene had not been characterized in the Rainbow Trout, we chose to use hybridization based cDNA library screening in an attempt to isolate the full-length expressed sequence. Briefly, plaque DNA from the cDNA library was fixed to nylon membranes to allow hybridization with a ^{32}P labeled probe. Following the pre-hybridization wash, the membranes were hybridized overnight at 42°C. Following the hybirdization, three washes of each membrane were used to remove unbound probe, these consisted of 2x SSC with 0.1% SDS at room temperature, 0.5x SSC with 0.1% SDS at 55°C, and 0.1x SSC with 0.1% SDS at 65°C. Following the washes, the membranes were exposed for at least 3 hrs to Bio-Max film (Eastman Kodak Co., New Haven, CT) at –80°C. Probe DNA was generated from the plasmid DNA isolated in the preliminary experiment. Following EcoRI restriction digestion of the plasmid DNA, the insert fragment (~1400 bp) was gel purified using the Freeze 'N Squeeze column method (Bio-Rad, Hercules, CA). This fragment was labeled with ^{32}P

using Amersham dCTP Ready-To-Go Labeling beads according to the manufacturer's protocol (Amersham Biosciences, Piscataway, NJ). Approximately 3.0 x 10^6 plaques were screened. Fifteen positive plaques, which had been subject to a second and a third rounds of screening, were isolated for sequence analysis.

Once the individual positive plaques were isolated, the phagemid vector (pBluescript) was excised using the ExAssist Helper Phage following Stratagene's protocol (Stratagene, La Jolla, CA). The resulting plasmid was sequenced from both forward and reverse directions, using the T7 and T3 promoter primers and the Big Dye Terminator Reaction (see above). A contig of the two sequences was constructed for each clone using GeneTools 1.0 (BioTools, Edmonton, AB, Canada). For clones not showing overlapping sequence, primer walking was preformed to allow determination of the full-length sequence.

Tissue Distribution of UCP3 in Rainbow Trout

Reverse transcription-polymerase chain reaction (RT-PCR) was used, in combination with Southern Blot analysis to determine the tissue distribution of the UCP3 in the Rainbow Trout. Total RNA was isolated from adult (~2 yr old) mixed-sex rainbow trout following the TRI-Reagent (Molecular Research Center Inc., Cincinnati, OH) modification of the guanidine isothiocyanate/phenol-chloroform method as described by Chomczynski and Sacchi (1987). The quantity of RNA will be estimated using the absorbance at 260 nm on a Shimadzu Spectrophotometer (Shimadzu Corp., Columbia, MD). The quality of the RNA was assessed by formaldehyde agarose gel electrophoresis with the visualization of the 28s and 18s rRNA bands. All RNA samples were stored at

–80°C. RNA was isolated from the brain, heart, liver, kidney, spleen, skeletal muscle, pituitary, stomach, intestines, eye, skin and adipose tissues. Reverse transcription was performed with 2 μg of total RNA, 2 μg of oligo-dT primers and Murine Maloney Leukemia Virus reverse transcriptase (MMLV-RT), as previously described (Kocamis et al., 2001). Taq polymerase (Display Systems, Los Angeles, CA) and gene specific primers were used for gene product amplification with the reaction set up as described above. A touchdown reaction was run with an initial annealing temperature of 68ºC, reducing by 1ºC for 10 cycles and then an additional 25 cycles with an annealing temperature at 58ºC. The products were separated by 1% agarose gel electrophoresis containing 200 ng/mL Ethidium Bromide and visualized and photographed with the FlouroChem Imaging System (Alpha-Innotech, Corp, San Leandro, CA). The identity of the products was verified by Southern Blot analysis.

Conservation of the UCP3 Ava III SINE in Other Salmonid Species

Genomic DNA samples were isolated for Rainbow Trout (Kamloop, Red Band, Cutthroat and Steelhead strains), Brook Trout, Brown Trout, Artic Char, Atlantic Salmon, and Pacific Salmon using Bio-Rad's AquaPure Genomic DNA Isolation Kit (Bio-Rad, Hercules, CA). This DNA was subject to PCR amplification for the AvaIII SINE located in the 3`-UTR of the Rainbow Trout UCP3 gene. The PCR reaction was setup as decribed above, using 1 μL of genomic DNA as the template. The PCR reaction consisted of 35 cycles with an annealing temperature of 60°C and primers specific to the SINE element in 3' UTR of UCP3. The upper primer (5'-GGGGACCTCTAGCCATTC-3') and lower primer (5'-TCAACTGAAATGTGTCCTCCGC-3') were designed to

amplify a 201-bp product of UCP3 3'-UTR including the AvaIII SINE. The products were separated by 1% agarose gel electrophoresis containing 200 ng/mL Ethidium Bromide and visualized and photographed with the FlouroChem Imaging System (Alpha-Innotech, Corp, San Leandro, CA). The identity of the products was verfied by southern blot analysis. The probe used for the Southern analysis was a sub-clone from the 3' UTR of the Rainbow Trout UCP3 cDNA labeled using the Ready-to-go Labeling beads (Amersham Biosciences, Piscatway, NJ) as previously described.

RESULTS AND DISCUSSION

Table 1 shows the identity of the three unique sequences isolated through EST and PCR screening of the RT Liver cDNA library. The 28S Ribosomal RNA (RTI#12) represents the original AvaIII SINE identified through the EST screening of the cDNA library. PCR screening and cloning subsequently lead to the identification of two additional unique sequences containing an AvaIII SINE (see Table 1). As such, these genes may be located in identifiable regions of the genomic DNA linked to evolutionary or phenotypic changes in Salmonids (Murata et al., 1996). To investigate this possibility further, we felt the potential role of UCP3 in metabolic regulation would make it an excellent candidate for association with phenotypic changes. However, the identification of a novel gene product (unknown in Table 1) containing a phylogenic marker could represent a more useful marker specific to Salmonids, a consideration that is currently being investigated in our laboratory. However, the role of UPC3 as a potential metabolic

regulator in the growing animal lead us to further investigate this previously unreported uncoupling protein from a cold water fish (Rainbow Trout).

Using a cloned partial sequence isolated by PCR screening of the liver cDNA library, we were able to identify 15 clones that hybridized specifically to the UCP3 probe. Thirteen of the 15 clones were found to contain the identical 1685 bp sequence (see figure 2) in which the longest open reading frame encodes for a 309-aa polypeptide with 69% aa homology to porcine (GenBank accession no. AAD33396) and canine (GenBank accession no. Q9N2I9) UCP3s. The cDNA identified showed the Rainbow Trout UCP3 (Figure 2) is transcribed as a 1685 bp mRNA consisting of a 101-bp 5' untranslated region, a 930-bp open reading frame, and a 654-bp 3'-untranslated region. We have identified the AvaIII SINE as being an 111-bp sequence located in the 3'-UTR. The two additional clones identified (UCP3v1 and UCP3v2) were identified as potential splice variants of the UCP3 gene. UCP3v1 contained three insertions, while UCP3v2 contained only one, which was identical in location in sequence to the second insertion in UCP3v1 (see Figure 2). The first insertion was found 126-bp downstream from the translation start codon, and was identified to be 133-bp in length. The second insertion was 134-bp in length and was located 334-bp downstream from the start codon, not including the first insert. The final inserted sequence was 370-bp long and was found 533-bp from the start codon. Following the GT-AG sequence found at the ends of each of the insertions (see Figure 2), we can conclude that these are the result of splice variations in the UCP3 gene. Following the open reading frame of the UCP3 cDNA, each of the variants would result in the translation of a truncated form of the protein. This may support a similar situation

as in humans in which a truncated form of UCP3 (UCP3S), results from splice variation and has been suggested to be a non-functional form of the protein (Solanes et al., 1997) .

The similarity in the aa sequence between the mammalian homologues (swine and dog) would suggest a similar structure for the UCP3 identified in the current study and therefore we would suggest, based on the homology, that the protein in the RT would have a conserved function. The next point was to determine the tissue distribution of UCP3 in the Rainbow Trout tissues. Using reverse transcription-polymerase chain reaction we found that UCP3 was expressed in a wide variety of tissues scanned (Figure 3). This was a somewhat unexpected result, as previous reports have shown UCP3 expression to be limited to skeletal muscle and BAT, with significantly lower expression in the cardiac muscle (for review see Fleury et al., 1997). In the present study, the greatest expression was found in the liver followed by the gastrointestinal organs (stomach and intestines), which may suggest a significantly different role for the UCP3 in metabolic regulation in the Rainbow Trout. In tuna and lamnid sharks, a heat exchange system surrounding the viscera has been suggested to have a key role in maintenance of elevated organ temperature increasing the digestive capabilities of the fish (for review see Carey et al., 1971). In a similar manner, we can suggest a similar role for the expression of UCP3 in and around the visceral organs of the rainbow trout, a theory that could be tested by comparing the expression of this gene in trout reared under a variety of temperatures.

A final consideration was the conservation of the AvaIII SINE element found in the 3'UTR of the Rainbow trout UCP3 gene. Using PCR and Southern Blot analysis, we were able to demonstrate the presence of this AvaIII SINE in a variety of salmonid

species (Figure 4). However, this investigation revealed two interesting results. First, we found in addition to the expected 201-bp fragment, found in all Rainbow Trout strains and the Pacific salmon, that a 178-bp fragment was found common to all the Salmonid species (see Figure 4). The Southern Blot data indicate that this fragment shares close homology to the 201-bp fragment, and thus may indicate the presence of a closely related gene in all salmonid species. Solanes et al. (1997) suggested based on the high homology and close localization on chromosome 11, that UCP3 resulted from a duplication event of UCP2. Although we have not specifically determined either the location of the gene or the identity of the sequence around the 178-bp band, this may indicate a similar event occurred in the salmonids. Additionally, in the Brook Trout, Brown Trout, Artic Char and Atlantic Salmon, in addition to the 178-bp band discussed above we found a 400-bp ampilcon associated with the lack of the 201-bp band. This raises the interesting possibility that there is an alteration in the 3' UTR of the UCP3 in these salmonid species. Although significantly beyond the scope of the present study, we could investigate the potential that this change may result in a significant alteration in metabolic efficiency when comparing these different salmonid species, assuming that this alteration leads to the change in the functionality of the UCP3 protein.

In the current study, we set out to identify the SINE elements found in the transcribed regions of the gene. However, this shortly lead into the identification of the UCP3 cDNA in the Rainbow Trout, and two splice variants. Based on the tissue distribution, we project this protein to have a role in the base maintenance of a core body temperature to allow normal function. Variation in the sequence and copy number of the AvaIII SINE in the salmonid species has allowed their use as a maker of genetic

varaiaton. Therefore, the relationship between this potential metabolic regulator and the phylogenetic marker may prove useful as a genetic marker of body partitioning or other marketable traits.

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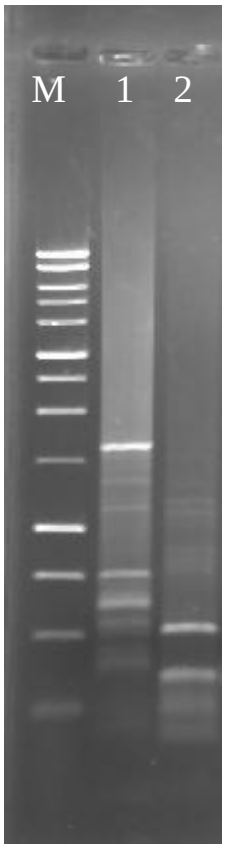

Figure 1- Determination of Amplicon size from the PCR screening of liver cDNA library with the AvaIII #6 Primer (see materials and methods) and either T3 (lane 1) or T7 (lane 2) promoter primer. Products separated on 1% agarose gel and stained with Ethidium Bromide.

Table 1- Expressed Sequences identified by PCR screening of liver cDNA library as containing the AvaIII SINE element, and length of the SINE element found in each transcript

Clone ID	Gene Identity	SINE Location	SINE Length
RTI#12	28s Ribosomal RNA		25 bp
Ava#6	Uncoupling protein 3	3'-UTR	121 bp
Ava#34	Unknown	3'-UTR	231 bp

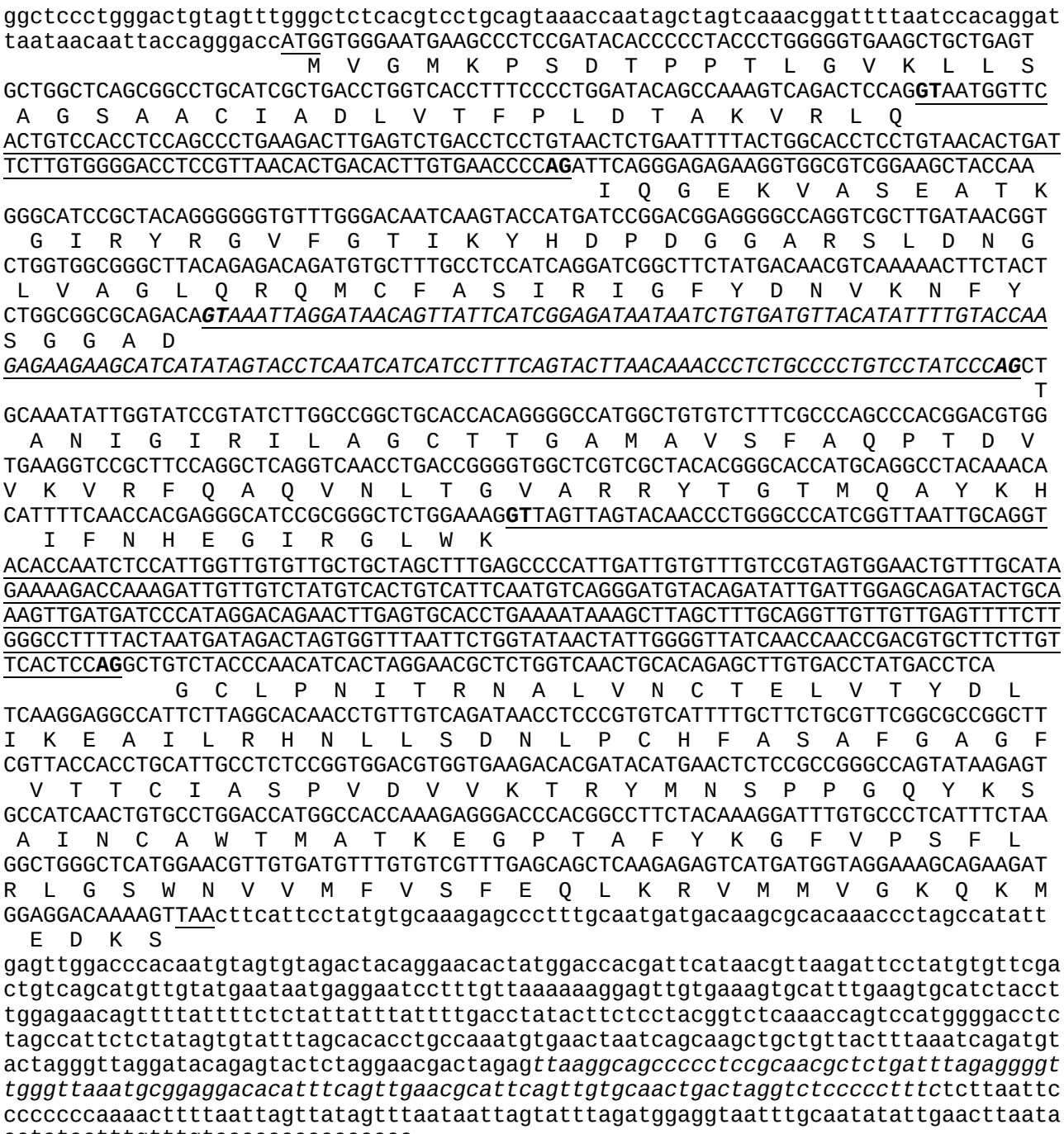

Figure 1 – cDNA sequence of Rainbow trout UCP3 with the translated 309 aa peptide sequence. Additionally, this figure shows the insertions included in UCP3v1 (Underlined and *Underlined Italic* Sequence) and UCP3v2 (*Underlined Italic Sequence*) and the location of the AvaIII SINE (*Italic Sequence*).

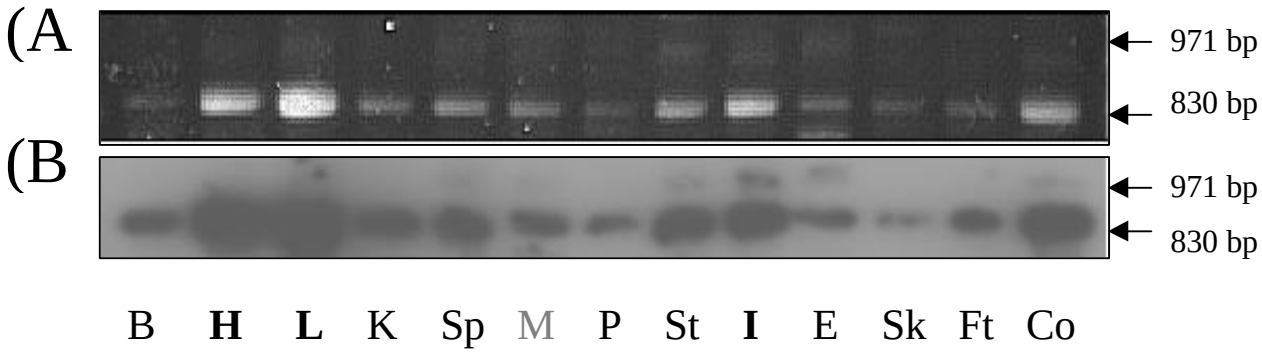

Figure 2 – UCP3 gene expression pattern in tissues from rainbow trout. This shows both the electrophoresis of PCR Products (A) and the Southern Blot analysis (B). UCP3 (830 bp) and UCP3v2 (971 bp) were the only transcripts detected in the tissues, with highest expression of UCP3 in the liver and the heart. UCP3v1 (1473 bp) transcript was not detected in the tissues screened. Tissues screened: B-Brain, H-Heart, L-Liver, K-Kidney, M-Skeletal Muscle, P-Pituitary, St-Stomach, I-Intestines, E-Eye, Sk-Skin, Ft-Fat Tissue, Co-Control Plasmid.

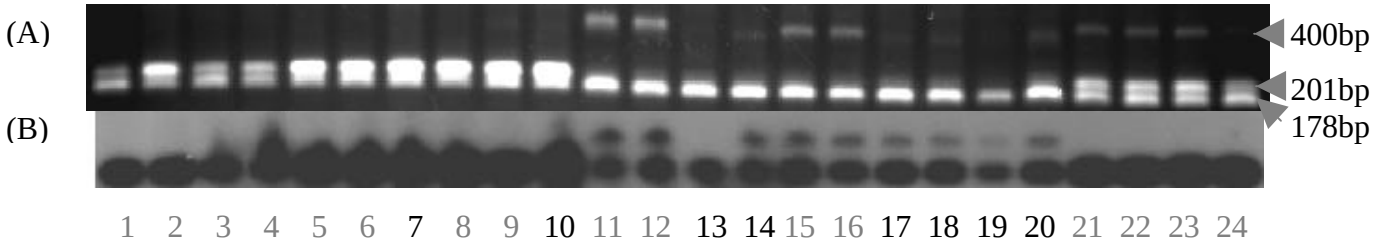

Figure 3 – Conservation of the AvaIII SINE element in the 3' UTR of the UCP3 transcripts among Rainbow Trout strains and other Salmonid Species as determined by Gel Electrophoresis of PCR Products (A) and Southern Blot Analysis (B). In addition to the expected 201 bp band, a 178 bp product common to all of the samples screened, and a 400 bp band was identified in a few samples. Samples: Lanes 1-6 – Rainbow Trout Kamloop, Lane 7 – Rainbow Trout Red Band, Lanes 8-9 – Rainbow Trout Cutthroat, Lane 10 – Rainbow Trout Steelhead, Lanes 11-12 – Brook Trout, Lanes 13-14 – Brown Trout, Lanes 15-16 – Artic Char, Lanes 17-20 – Atlantic Salmon, Lanes 21-24 – Pacific Salmon.

Appendix 3

Activin-B, Activin Type-I and –II Receptors, and Follistatin Gene Expression in MCF-7 and MDA-MB-231 Breast Tumor Cell Lines

ABSTRACT

Background: Activins are members of the transforming growth factor beta (TGF-β) family and are known to modulate the proliferation and differentiation of several cell lines. Follistatins, monomeric glycosylated proteins, specifically neutralize the biological actions of activins as well as other members of the TGF-β family. The objective of the present study was to investigate the relationship between TGF-β sensitivity and gene expression of activin-B, activin receptor type-I and -II, follistatin and myostatin in the TGF-β-sensitive (MDA-MB-231) and -resistant (MCF-7) breast cancer cell lines.

Materials and Methods: Cell cultures were maintained in 7% CO_2 in DMEM + 10% FBS and gentamicin. Reverse transcription polymerase chain reaction (RT-PCR) was used to determine the expression profile of the aforementioned genes.

Results: Activin-B, receptor type–I, and follistatin gene expression were higher in the MDA-MB-231 than in MCF-7 cell line, whereas receptor type-II expression was similar in both cultures. We also examined the gene expression level of myostatin, a recently identified TGF-β family member, and myostatin mRNA was not identified in either cell line.

Conclusion: The expression profile of activins, their receptors and binding protein (follistatin) indicate a potential relationship between the TGF-β sensitivity and expression of these genes in the investigated cell lines.

Keywords: Activin, Activin Receptors, Follistatin, MCF-7 Cells, MDA-MB-231 Cells

Abbrevations: TGF-β = Transforming Growth Factor-β, RT-PCR = Reverse Transcription-Polymerase Chain Reaction, ER = Estrogen Receptor

INTRODUCTION

Activins, members of the Transforming Growth Factor-β (TGF-β) family, exist as three distinct β subunits (βA, βB, βC) in humans. However, only βA and βB subunits have been shown to form biologically active dimers (βAβA, βBβB and βAβB) [1]. Activins function through TGF-β family receptors (activin receptors), type-I and type-II [2]. Activins bind to the type-II receptor forming a complex, which then recruits the type-I receptor to initiate the serine/threonine kinase signal transduction pathway [1]. Follistatins, functioning as activin binding proteins, are glycosylated monomers resulting from alternate splicing (288 and 315 aa forms) and proteolytic modification (300 aa form) of a single gene transcript [3]. Follistatins bind to the β-subunits of the activin molecules and thereby attenuate their activity [1]. Both activins and follistatins have been found in a number of tissues and are thought to interact in an autocrine/paracrine fashion during development [4].

Altered activin/follistatin gene and protein expression have been reported in ovarian epithelial carcinomas [5], human pituitary adenoma [6], and prostastic cancer cell lines [7], indicating a potential role for the activin/follistatin system in development or progression of cancer. However, the effect of activins on tumor cell growth seems to diverge among different cell types. For example, activins are mitogenic for embryocarcinoma P19 cells, while inhibiting profileration for the Y-79 rentinoblastoma

cells [1]. The role of activins, their receptor and follistatin in the regulation of breast cancer development is possibly limited by the sensitivity of the cells. The MDA-MB-231 (estrogen receptor (ER) negative) cells have been shown to be more sensitive to TGF-β family members than the MCF-7 (ER-positive) cell line [8,9,10]. Additionally, the effects of estrogens [10] and progestins [11] on breast cancer cell lines were positively correlated to reduced TGF-β family expression.

Myostatin, a novel member of the TGF-β family, has been proposed as a negative regulator of muscle cell proliferation [12] and could play a role in the regulation of tumor cells. Additionally, Ji *et al.*, [13] found significant myostatin expression in the lactating mammary glands of pigs. Therefore, a potential relationship between myostatin and breast tumor development may be postulated.

Members of the TGF-β family have been shown to inhibit proliferation of breast cancer cell lines [10]. Therefore, profiles of endogenous TGF-β family gene expression may provide a better understanding of and development of better breast cancer treatments. In the current study, we investigated the relationship between TGF-β sensitivity and the gene expression profiles of activin-B, activin receptor type-I and type-II, follistatin and myostatin in MCF-7 and MDA-MB-231 breast tumor cells.

MATERIALS and METHODS

Cell Culture

Permanent cell lines derived from a human breast carcinoma were used in the current study. MCF-7, between passage numbers 40 and 50 and MDA-MB-231, between passage numbers 15 and 60, were maintained in Dulbecco's Modified Eagle's Medium

(DMEM) (Bio Whittaker, Rockland, ME) supplemented with 10% fetal bovine serum (FBS), and 40 μg/ml gentamicin. The cells were maintained at 37°C in a humidified atmosphere of 93% air and 7% CO_2. As the cells approached confluence, they were passaged weekly at a 1:5 ratio (MCF-7) and 1:10 ratio (MDA-MB-231).

RT-PCR

RNA isolation and RT-PCR were performed as previously outlined [14]. Briefly, 50 μL of the PCR cocktail containing: 5 μL of 10x Taq buffer (1.5 mM $MgCl_2$ final concentration), 1 μL dNTPs (final concentration 0.2 mM each), 1 μL of sense and anti-sense primers (1 mM final concentation), 1 μL of Taq DNA Polymerase (Display Systems Biotech, Vista, CA) and 41 μL of nuclease free water to 2 μL of the reverse transcription reaction. The PCR started with one cycle consisting of denaturing (94°C) for 5 min, annealing (65°C for activin-B, 60°C for activin type-II receptor, 55°C for β-Actin) for 1 min, and extension (72°C) for 1½ min. The first cycle was followed by 30 cycles consisting of 1 min intervals of denaturing at 94°C followed by annealing at (65°C activin-B, 60°C for activin type-II receptor, 55°C for β-actin followed by extension at 72°C. These cycles were followed by a final 10 min extension step (72°C). For follistatin and activin type-I receptor, touchdown PCR was run. This program consisted of a 5-min denaturation step (94°C), followed by 5 cycles in which the initial annealing temperature of 72°C was reduced by 1°C per cycle, then 30 cycles in which the annealing temperature was 68°C. Again the reaction was concluded with a 10 min extension step at 72°C. Gene specific primers (Table 1) were designed based on published sequences for each gene, and obtained from Gibco BRL Inc. (Grand Island, NY). All reactions were run on a PTC-200 thermocycler (MJ Research, Watertown, MA). A water control (no

cDNA) reaction was run with each cocktail to assure there was no contamination of the components of the reactions. The identity of the PCR products was verified by sequence analysis.

The amplified gene products were visualized by electrophoresis on 1.5% agarose gels stained with ethidium bromide (0.2 mg/mL). The gene products were photographed under UV light using FluorChem™ Imaging System (Alpha-Innotech Corp., San Leandro, CA) and quantified by densometric analysis of the photograph using FlourChem™ Software (Alpha-Innotech Corp., San Leandro, CA). The densitometric values for each gene product were normalized to densitometric values of the β-Actin housekeeping gene.

Statistical Analysis

Analysis of Variance was performed using the GLM procedure of SAS® (SAS Institute, Cary, NC). Means for each gene were compared between the two cell lines. For all analyses, statements of significance were based on $P<0.05$ unless otherwise noted.

RESULTS

β-Actin expression levels did not differ between the two cell lines (data not shown) and therefore was deemed suitable for normalization of the densitometeric values. As shown in figure 1, activin-B expression was observed in both the MCF-7 and MDA-MB-213 cells lines, and the level was higher in the MDA-MB-231 cells than in the MCF-7 cells ($P<0.01$). In both the MDA-MB-231 (ER -) and MCF-7 (ER +) cell lines, similar expression of the activin type-II receptor was found (figure 2). However, we found

higher expression of the activin type-I receptor in the MDA-MB-231 cell line (figure 3, $P<0.03$). Follistatin expression was determined to be higher in the MDA-MB-231 cells as compared to the MCF-7 cells ($P<0.03$, figure 4). We were unable to detect any myostatin expression in either cell line (data not shown).

DISSCUSSION

Jeng *et al.* [10] demonstrated a difference regarding TGF-β family member sensitivity among different breast tumor cell lines. Specifically, they indicated that the MDA-MB-231 cells were less sensitive to inhibition of proliferation by TGF-β members than the MCF-7 cells. This difference could be attributed to receptor limitations in the cell lines, i.e. gene mutations or reduced expression [9]. Liu et al [15] showed that both ER+ (MCF-7) and ER- (MDA-MB-231) cell lines expressed the activin type-II receptor as reflected in the current study. We showed a significant reduction in the expression of the activin receptor type-I in MCF-7 cells. Suggesting that MCF-7 cells may be less sensitive to activin treatment than MDA-MB-231 cells. However, it has been found that activin-A reduced proliferation and altered colony morphology of the MCF-7 cells [15]. With respect to the MCF-7 cell line, it was determined that higher passage numbers reduced TGF-β sensitivity [10]. Thus, the incongruity between the previous reports [10,15] and the current study may be related to the passage numbers of the cells studied. The current experiment used a moderate passage number (40-50). Therefore, we should not overlook the possibility that the loss of the activin receptor type-I expression may be the result of the increased passage number.

A number studies have shown the co-expression of activin-B and follistatin in a variety of cell culture systems [for review see 1], consistent with the cell lines investigated in the current study. Additionally, both in vitro and in vivo studies show that exogenous activin treatment lead to increased follistatin expression [for review see 2]. We may therefore infer that the increased follistatin expression in the MDA-MB-231 cells may be the response to the higher levels of endogenous activin-B. Attenuation of follistatin in the MCF-7 cells would be expected because these cells showed not only a lower activin-B expression, but also lower receptor type-I expression. Collectively, these data indicate that regulatory interactions between activins and follistatin appear to exist in these breast tumor cell line.

CONCLUSION

The expression of activins, their receptors and one of their binding proteins (follistatin) in both an ER (+) (MCF-7) and ER (-) (MDA-MB-231) breast cancer cells lines was investigated. We conclude that the expression of activin-B, activin receptor type-I and follistatin are different between the two cell lines. The expression profiles of activins, their receptors and binding proteins (follistatins) indicate a potential explanation for the differences in TGF-β sensitivity of the investigated cell lines. Thus, the role of activins and follistatins in the development and progression of breast cancers should be considered when evaluating novel treatments. Additionally, we showed that the regulatory interactions between the activins and follitsatins appeared to be intact, and therefore may be useful in evaluating both the type of cancer and prospective treatments.

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Appendix 4

IGF-I, IGF-II AND IGF-RECEPTOR-1 TRANSCRIPT AND IGF-II PROTEIN EXPRESSION IN MYOSTATIN KNOCKOUT MICE TISSUES

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ABSTRACT

Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) and immunohistochemistry were performed to demonstrate whether a correlation exists between insulin-like growth factors, positive regulators of growth, and myostatin, a negative regulator of muscle growth. IGF-I, -II and IGF-receptor-1 mRNA (IGF-R1) and IGF-II protein expressions were determined in control and myostatin knockout mice tissues. IGF-I gene expressions were similar between control and knockout mice tissues, whereas IGF-II mRNA levels were significantly higher in myostatin knockout mice kidney and soleus muscles than those of control mice ($P < 0.01$). IGF-R1 mRNA levels from control mice heart ($P < 0.05$) and kidney ($P < 0.01$) were significantly higher than in myostatin knockout mice, whereas levels were lower in pectoralis muscle of control mice than knockout mice ($P < 0.01$). The strongly IGF-II positive cells in soleus muscle were more common in myostatin knockout mice and were seen in a few foci in control mice. IGF-II immunoreactivity in both control and myostatin knockout mice kidneys was localized to the epithelium of renal tubules and collecting ducts. Reciprocal changes in the expression of myostatin and IGF-II and IGF-R1 may facilitate the better understanding not only of skeletal muscle growth but also of other organ development in mammalians, as well as of the pathophysiology of these tissues associated with disease.

INTRODUCTION

Insulin-like growth factors (IGF-I and -II) are produced by several different tissues and are essential for both embryonic and postnatal development.[5,13] 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 reduced development of muscle tissue,[18] whereas transgenic mice overexpressing IGF-I showed enhanced body growth with an increase in muscle mass.[14] Additionally, IGF-II expression has been positively associated with skeletal muscle development in double-muscled cattle [11] and in pig breeds with exceptional muscle mass. [16] In vitro, both IGFs inhibited apoptosis [22] and promoted proliferation and differentiation of skeletal muscle cells.[1] 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-β family, was disrupted by homologous recombination in mice, skeletal muscle mass significantly increased, up to three times the normal size.[15] 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-muscled cattle breeds.[8,10] Therefore, it has been proposed that myostatin is 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-1 mRNA (IGF-R1) and protein levels were determined in a wide variety of myostatin knockout mice tissues.

MATERIALS and METHODS

Animals

Nine-month-old male myostatin knockout (n= 3, SVJ/129) and control mice (n= 3, C57Bl/6) 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.[4] 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.

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_2O in a final volume of 15 μl. The samples were heated at 70^o C for 5 minutes and then immediately cooled to 4^o 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^{o} C for 1 hr followed by a 5 min incubation at 95^{o} C. For the PCR reaction, 2 μl of RT reaction mixture were added to 50 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_2O. The PCR reaction started with one cycle consisting of 94^{o} C for 5 minutes, an annealing step of 65^{o} C for IGF-I or 55^{o} C for IGF-II and β-actin for 1 minute and extension at 72^{o} C for 1 minute. The first cycle was followed by 30 cycles (25 cycles for β-actin) consisting of 45-s intervals of 94^{o} C, followed by 65^{o} C for IGF-I or 55^{o} C for IGF-II and β-actin, followed by 72^{o} C. For IGF-RI, touchdown PCR was run. This program consisted of a 5-minute, 94^{o} C denaturation step, followed by 5 cycles in which the initial annealing temperature of 72^{o} C was reduced by 1^{o} C per cycle, then 30 cycles in which the annealing temperature was 68^{o} C. 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.[2]

The sequence of the forward primer was 5 GCTGAGCTGGTGGATGCTCTTCAGTTC 3, and 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.[19] 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, and the reverse primer was 5 ACGTTTGGCCTCTCTGAACTCTTTGAG 3. Primers for IGF-R1 were designed on the based of published sequence of mouse IGF-I.[21] The sequence of the forward primer was 5 GACATCCGCAACGACTATCAG 3, and the reverse primer was 5 GTAGTTATTGGACACCGCATC 3. Primers for IGF-R1 amplified a PCR product of 395 bp that corresponded to bases (114-509) of the sequence. Forward and reverse primers for β-actin were predicted to amplify a 285 bp product as previously published,[24] as an internal standard to verify the level of amplification. The sequence of the forward primer was 5 TCATGAAGTGTGACGTTGACATCCGT 3, and the reverse primer was 5 CCTAGAAGCATTTGCGGTGCACGATG 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 semiquantitative. 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 μm and incubated at 60^{o} C for 20 minutes. Sections were then deparaffinzed 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. The coplin jar was placed in microwave on high for 1 minute, 45 seconds to bring temperature up to boiling point. 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. Slides were incubated in 3% hydrogen peroxide for 10 minutes at room temperature. After rinsing with distilled water, slides were incubated in Tissue Conditioner (Biomeda, Foster City, CA) for 10 minutes at 37^{o} C. 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 the section for 1 hour at room temperature. Slides were then rinsed with distilled water and incubated in tris buffered saline (TBS) for 5 minutes at room temperature. Vectastain biotinylated secondary antibody (anti goat IgG) was applied as outlined by the manufacturer (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature. 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 room temperature. Slides were rinsed with distilled water and incubated in for 5 minutes at room

temperature. Aqueous hematoxylin (Biomeda, 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 (Biomeda, Foster City, CA). After crystal mount was dried, they were post-mounted with Permount (Fisher Scientific, Pittsburgh, PA) and a glass coverslip was placed over the Permount.

Whole section digital images were captured using a Sprint Scan slide scanner (Polaroid, Cambridge, MA) and a PathScan Enabler (Myer Instruments, Houston, TX). Digital photomicrographs were captured using a Quantix digital camera (Photometrics, Tucson, AZ). For digital capture, images from control and knockout mice were captured in the same session using identical settings. 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

Probability of differences of least-square means was used to compare densitometric values (General Linear Model procedure of SAS, SAS Institute Inc., Cary, NC).

RESULTS

β-Actin, IGF-I, IGF-II and IGF-R1 mRNA Expression

β-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, and no IGF-I mRNA was detected in either control or knockout mice soleus muscles (Fig. 2). IGF-II mRNA levels were significantly higher in kidney and soleus muscles of myostatin knockout mice than control mice ($P < 0.01$, Fig. 3). No IGF-II gene expression was observed in liver of control and 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 from myostatin knockout mice, whereas levels were lower in pectoralis muscle of control mice than 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 immunohistchemistry 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 more 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 IGF–II are well-known positive regulators of muscle growth, their gene expressions in the current study demonstrated different patterns between muscle tissues of myostatin knockout and control mice. 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), but IGF-R1 mRNA levels from pectoralis muscles were significantly higher in myostatin knockout mice than control mice (Fig. 4). By contrast, IGF-II mRNA levels from soleus muscles were higher in mysotatin knockout mice than control mice, and 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 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 than soleus muscle of normal adult mice,[3] 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. (20) 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, whereas no change in IGF-I mRNA level was observed.[12] Based upon these in vitro and in vivo findings, it is 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,[7] the significance of increased IGF-R1 expression without corresponding increases of ligands from pectoralis muscles of myostatin knockout mice remains to be further explored. Additionally, the local or systemic production of the IGF binding proteins may play a critical role in regulating the activity of these growth factors and therefore merits further investigation.

In the present study, strong IGF-II gene expression was observed in myostatin knockout mice kidney, whereas IGF-R1 expression was significantly lowered when compared to control mice (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, as 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.[23] Although myostatin knockout kidney weight was not measured in the current and previous studies,[15] increased IGF-II levels may have caused the decline in IGF-R1 mRNA expression to maintain proper size of myostatin knockout mice kidney. Several studies, however, have suggested that metabolic actions of IGF-II, unlike its mitogenic actions, are not mediated through IGF-R1 but through insulin receptors, to which IGF-II can bind with low affinity.[6,9] Additionally, IGF-II, but neither insulin nor IGF-I, stimulated Na^+-H^+ exchange across the brush-border membrane of proximal tubular cells.[17] Therefore, it is possible 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 and growth hormone should be conducted to clarify this speculation.

Because tissue samples were obtained only at one 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 mammalians as well as pathophysiology of these tissues associated with disease.

Acronyms:

IGF – Insulin-like Growth Factor
IGF-R1 – Insulin-like Growth Factor-Receptor-1

PCR – Polymerase Chain Reaction
RT – Reverse Transcription
TBS – Tris Buffered Saline

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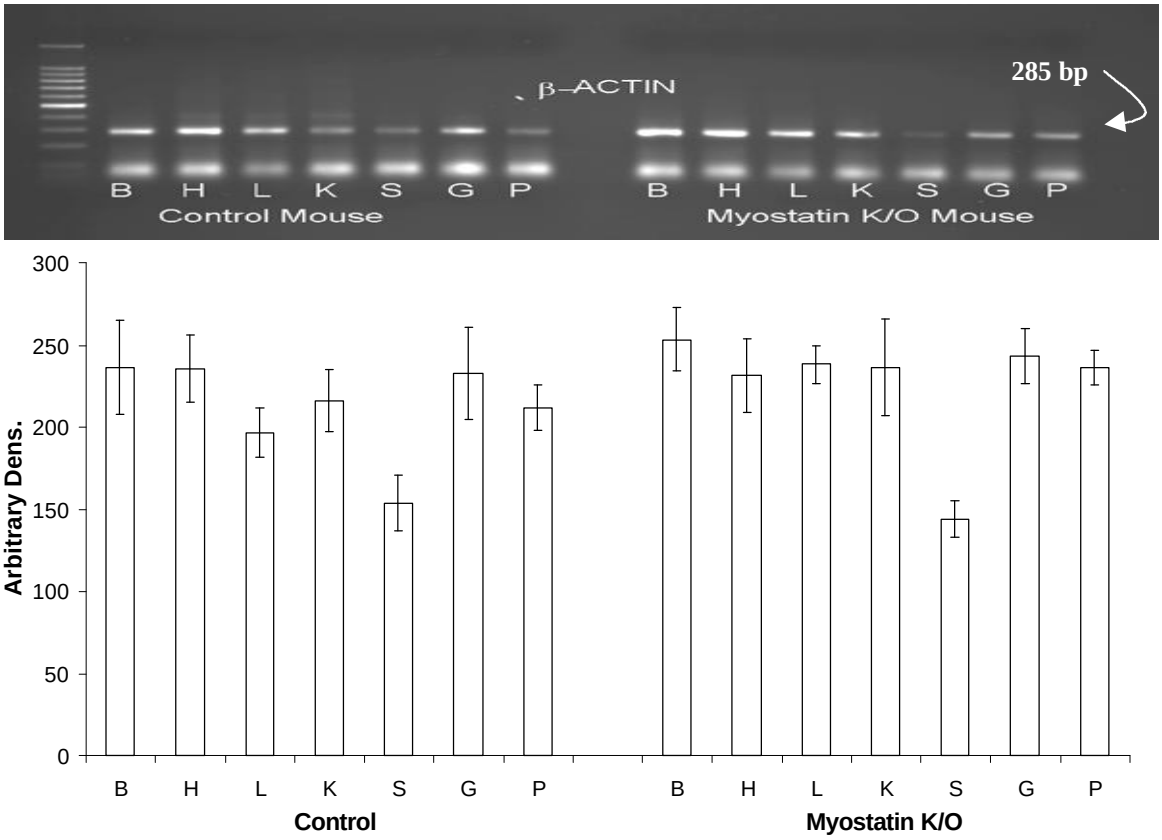


Figure 1. Steady-state levels of β-actin mRNA in brain (B), heart (H), liver (L), kidney (K), soleus (S), gastrocnemius (G), and pectoralis (P) muscle of control (n= 3) and myostatin knockout mice (n= 3) tissues. The bands for β-actin were analyzed by densitometry and the integration values (mean ± SD)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

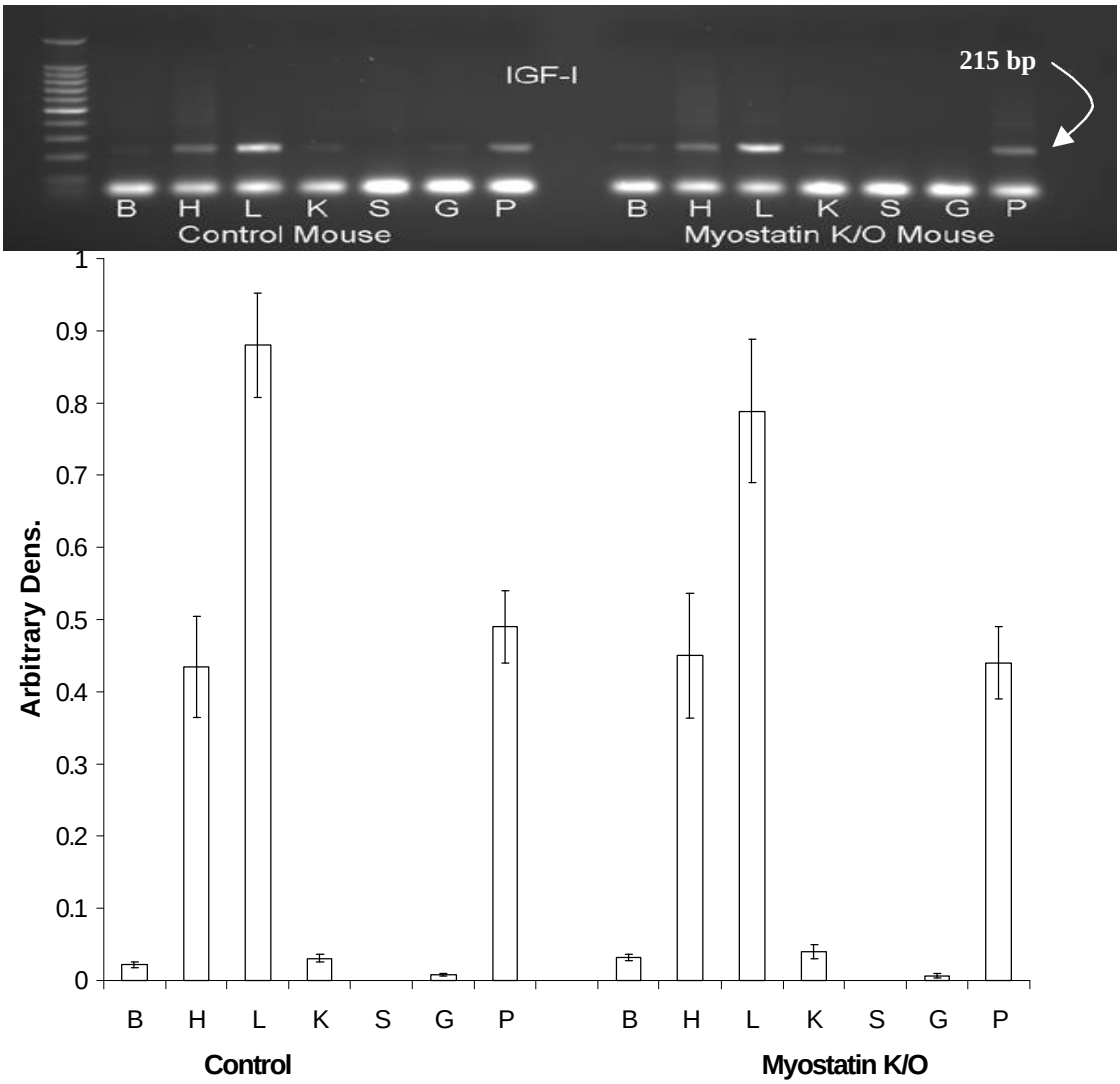


Figure 2. Steady-state levels of IGF-I mRNA in brain (B), heart (H), liver (L), kidney (K), soleus (S), gastrocnemius (G), and pectoralis (P) muscle of control (n= 3) and myostatin knockout mice (n= 3) tissues. The bands for IGF-I were analyzed by densitometry and the integration values (mean ± SD),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

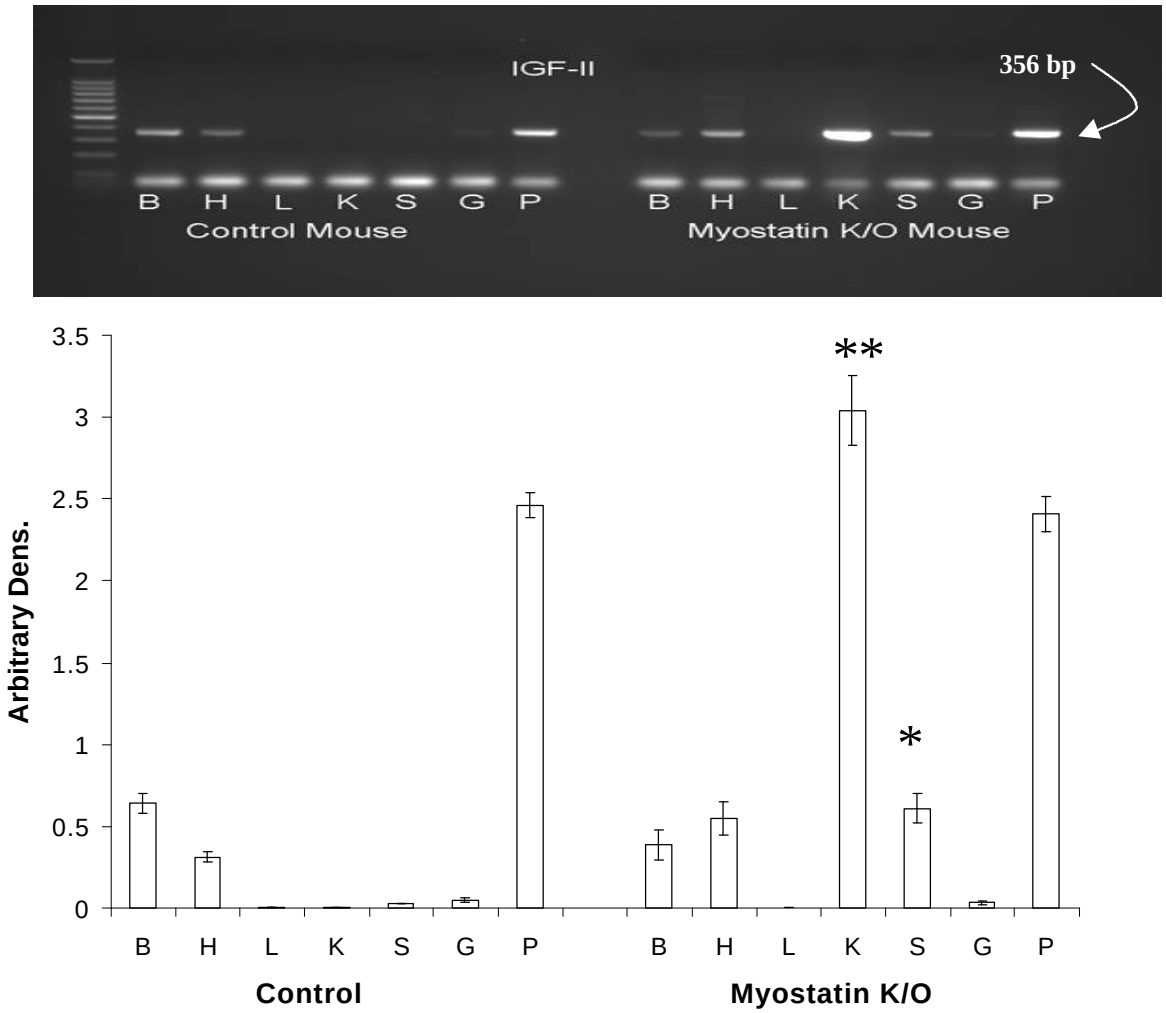


Figure 3. Steady-state levels of IGF-II mRNA in brain (B), heart (H), liver (L), kidney (K), soleus (S), gastrocnemius (G), and pectoralis (P) muscle of control (n= 3) and myostatin knockout mice (n= 3) tissues. The bands for IGF-II were analyzed by densitometry and the integration values (mean ± SD),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

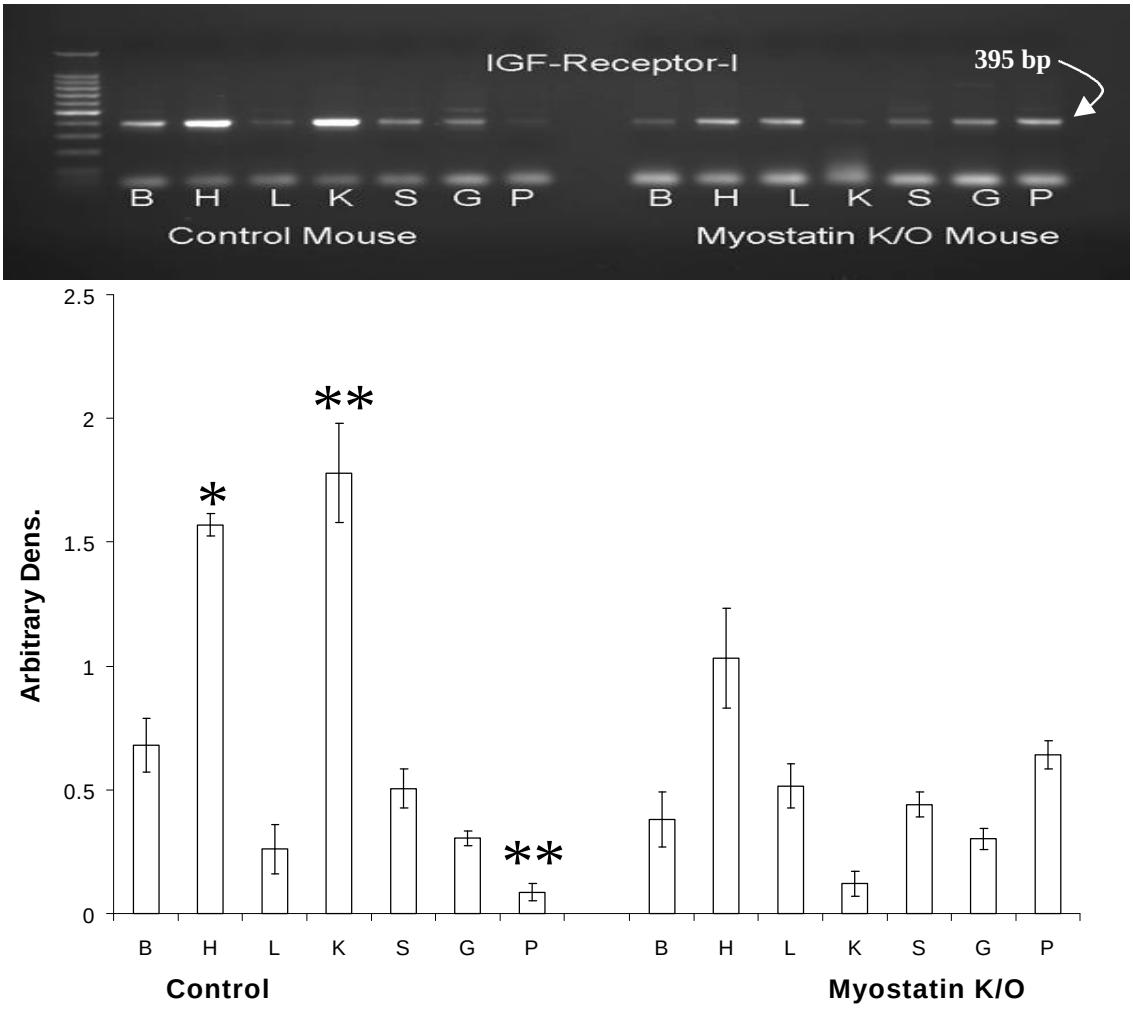


Figure 4. Steady-state levels of IGF-R1 mRNA in brain (B), heart (H), liver (L), kidney (K), soleus (S), gastrocnemius (G), and pectoralis (P) muscle of control (n= 3) and myostatin knockout mice (n= 3) tissues. The bands for IGF-R1 were analyzed by densitometry and the integration values (mean ± SD),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

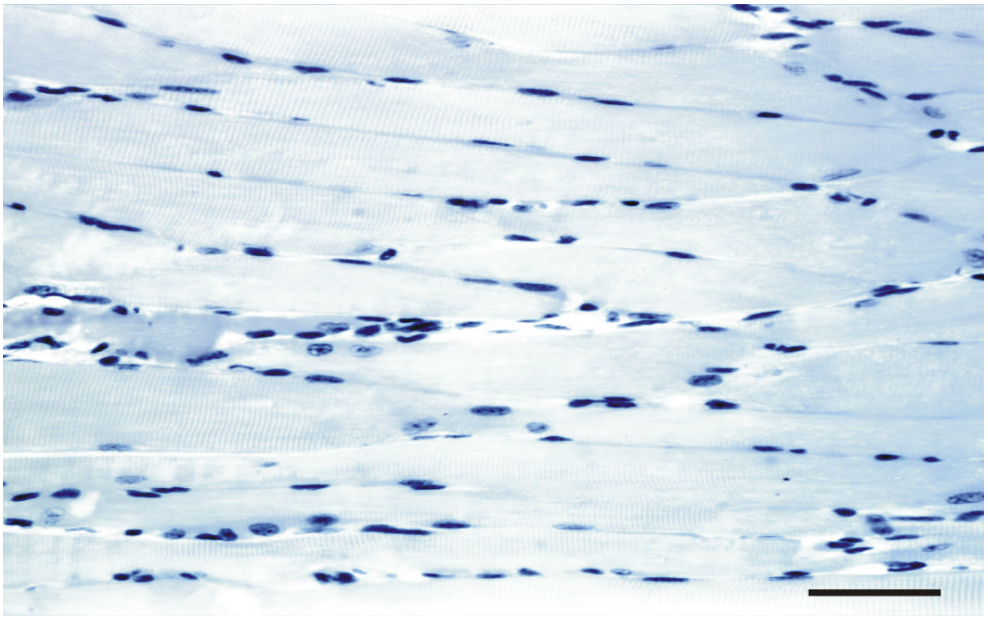
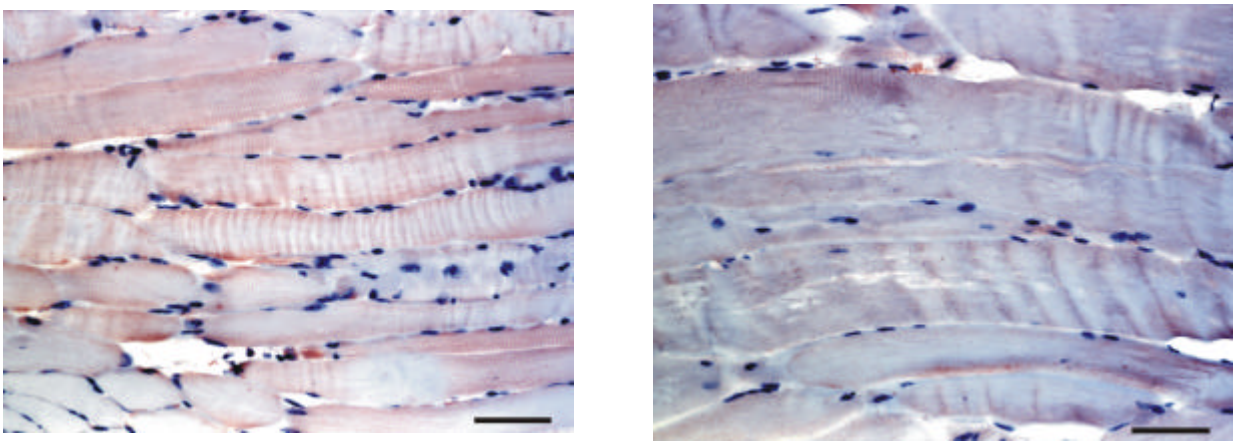


Figure 5. Rabbit Super Sensitive Control Serum (Biogenex, San Ramon, CA) in place of primary antibody was used as a negative control on one section for each run. Note the absence of red staining. Bar = 50 micrometers.

Control



Myostatin knockout

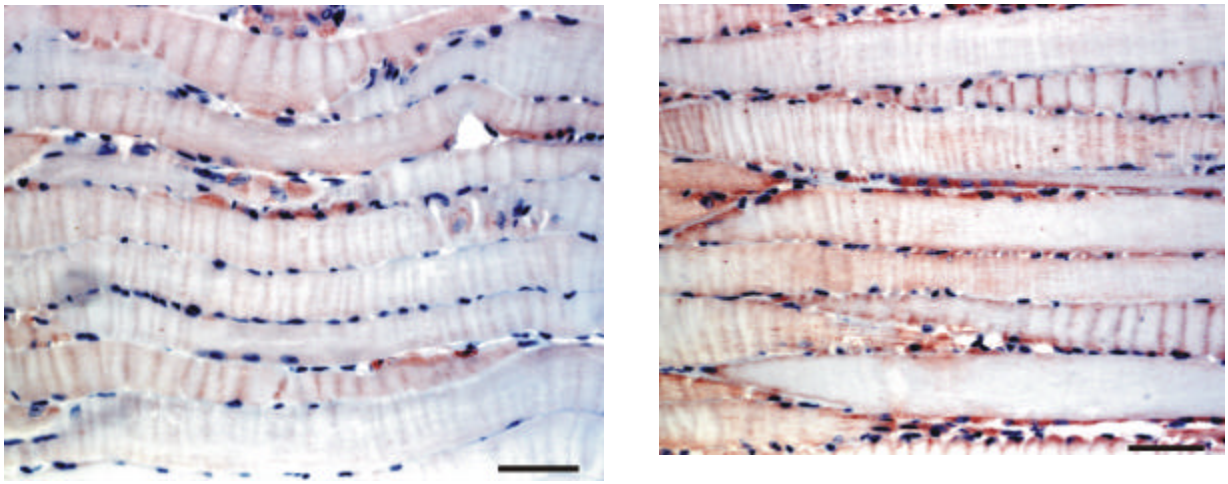


Figure 6. IGF-II immunohistochemistry in control and myostatin knockout mice soleus muscles. IGF-II immunoreactivity (red staining) was principally localized satellite cells. Bar = 50 micrometers.

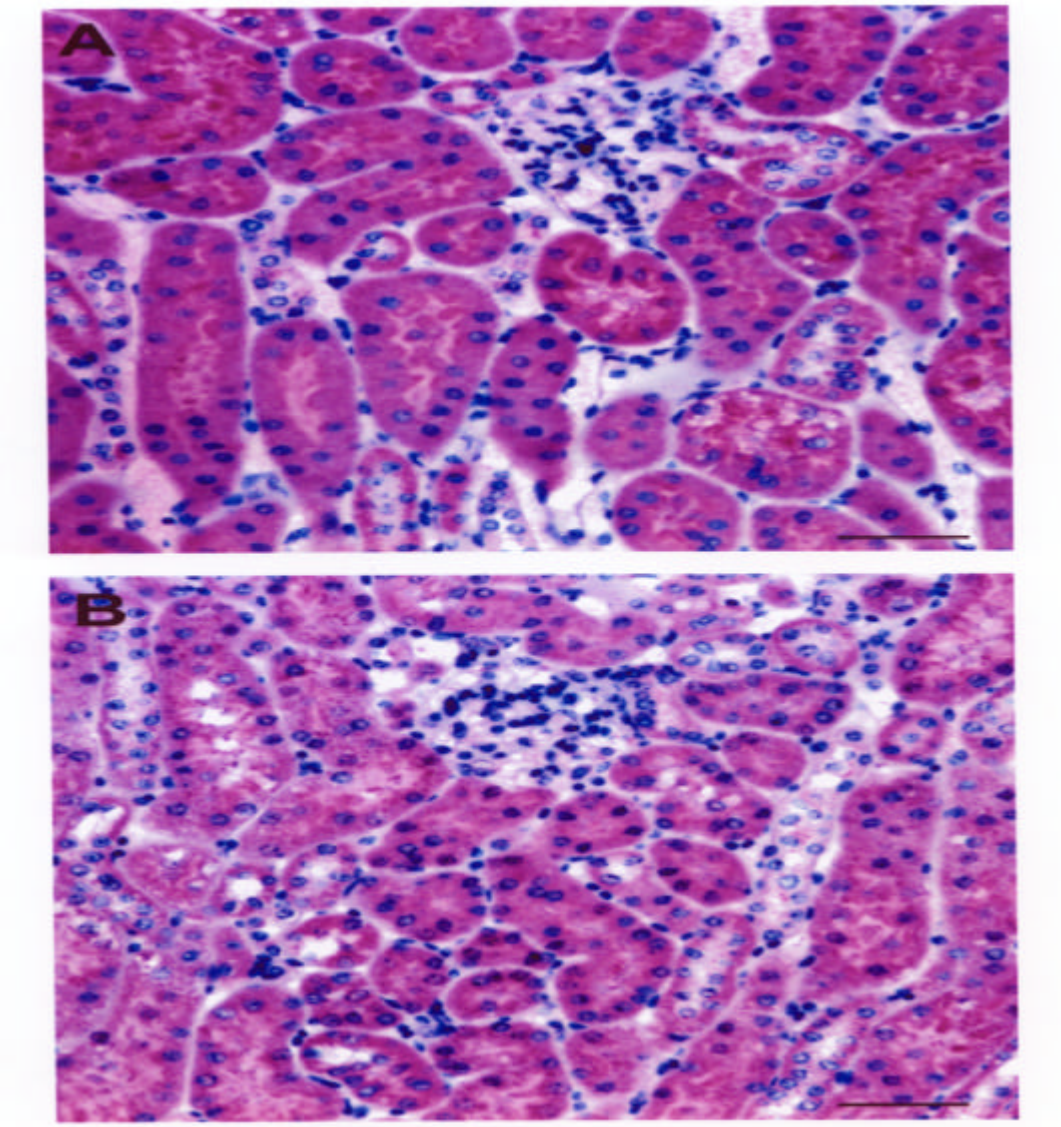


Figure 7. IGF-II immunohistochemistry in control (A) and myostatin knockout mice (B) kidneys. IGF-II immunoreactivity (red staining) was localized to the epithelium of renal tubules and collecting ducts. Bar = 50 micrometers.