PRODUCTION AND CHARACTERIZATION OF POLYCLONAL ANTIBODIES AGAINST CHICKEN MYOSTATINS

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ABSTRACT

Myostatin is a new member of the transforming growth factor-beta superfamily of secreted growth and differentiation factors. Myostatin is almost exclusively expressed in skeletal muscle and act as a negative regulator of skeletal muscle growth. Because anti-myostatin antibodies will be useful in investigating the mechanism of action of myostatin, an experiment was designed to produce polyclonal anti-chicken-myostatin antibodies. A PCR amplified prepro-myostatin composed of the prodomain plus mature myostatin and a C- terminal fragment containing only the mature myostatin were cloned into an expression vector, and these proteins were expressed in E. coli. The recombinant proteins were fractionated by SDS-PAGE, then the myostatin bands were cut out and electro-eluted to obtain purified myostatins. Rabbits were immunized with the purified myostatins to produce polyclonal anti-myostatin antibodies. IgGs were separated from the rabbit sera using Protein A affinity chromatography. Antibody binding characteristics were then examined using Western blot analysis of various chicken tissues. Both polyclonal antibodies demonstrated a strong affinity to both form of recombinant myostatin in Western blot analysis. The anti-mature myostatin antibody showed binding affinity to proteins at 50, 30 and 20 kD in skeletal muscle and liver. No specific binding in skeletal muscle was demonstrated by the anti-mature myostatin antibody, suggesting that the above proteins are non-myostatin proteins that have affinity to anti-mature myostatin antibody. In contrast, the anti-prepro-myostatin antibody showed affinity to a 37 kD band only in skeletal muscle in addition to the 50, 30 and 20 kD bands. Since the molecular weight of the myostatin prodomain is known to be close to 37 kD, it is postulated that the 37 kD protein specifically recognized by the anti-prepro-myostatin in skeletal muscles is likely to be a myostatin prodomain with further
validation of its binding specificity, the anti-prepro-myostatin antibody generated in this study will potentially be useful in investigating the mechanism of action of myostatin.
TABLE OF CONTENTS

ACKNOWLEDGEMENT ................................................................. I
ABSTRACT .................................................................................. II
LIST OF TABLE ........................................................................... VII
LIST OF FIGURES ........................................................................ VIII
CHAPTER 1 .................................................................................... 1
LITERATURE REVIEW .................................................................... 1
1.1 SKELETAL MUSCLE GROWTH AND DEVELOPMENT .................. 1
  1.1.1 Embryonic development of skeletal muscle ......................... 1
    1.1.1.1 Origin of skeletal muscle cells during embryogenesis ....... 1
    1.1.1.2 Role of molecular factors in somite development .......... 2
    1.1.1.3 Myoblast proliferation and differentiation ................... 4
      1.1.1.3.1 Myogenic regulatory factors ................................. 5
      1.1.1.3.2 Signals regulating MRF expression ....................... 7
  1.1.2 Postnatal skeletal muscle growth ...................................... 11
    1.1.2.1 Muscle fiber number ................................................ 11
    1.1.2.2 Satellite cells ......................................................... 12
1.2 MYOSTATIN, A NEGATIVE REGULATOR OF SKELETAL MUSCLE GROWTH ................................................................. 12
  1.2.1 Biochemical characteristics of myostatin ............................ 13
  1.2.2 Genetics of myostatin ....................................................... 16
  1.2.3 Physiological action of myostatin ..................................... 17
    1.2.3.1 Role of myostatin in the control of myoblast proliferation and differentiation ................................................. 17
1.2.3.2 Role of myostatin in the control of post-natal muscle growth ........................................... 19

1.2.3.3 Role of myostatin on adipose tissue metabolism ......................................................... 23

1.2.4 Regulation of myostatin signaling ................................................................................. 24

CHAPTER 2 ......................................................................................................................... 32

PRODUCTION AND CHARACTERIZATION OF POLYCLONAL ANTIBODIES AGAINST CHICKEN MYOSTATINS

2.1 INTRODUCTION ............................................................................................................. 32

2.2 MATERIALS AND METHODS ......................................................................................... 34

2.2.1 Amplification of chicken myostatin cDNAs ............................................................... 34

2.2.2 Construction of myostatin fragment expression vector .............................................. 35

2.2.3 Expression of recombinant myostatins ...................................................................... 35

2.2.4 Isolation of inclusion bodies .................................................................................... 36

2.2.5 Purification of recombinant protein .......................................................................... 36

2.2.6 Immunization of rabbit and serum collection ............................................................ 37

2.2.7 Enzyme-linked immunosorbent assay (ELISA) ........................................................ 37

2.2.8 Affinity purification of anti-myostatin IgG ................................................................. 38

2.2.9 Protein assay ........................................................................................................... 38

2.2.10 Sodium dodecyl sulphate and polyacrylamide gel electrophoresis (SDS-PAGE) analysis .................................................................................................................. 38

2.2.11 Western blot analysis ............................................................................................... 39

2.3. RESULTS ..................................................................................................................... 40

2.3.1 Expression of recombinant chicken myostatins ....................................................... 40

2.3.2 Purification of recombinant chicken myostatins ....................................................... 40
2.3.3 Production of antibodies against chicken myostatins ........................................... 40
2.3.4 Affinity purified anti-myostatin IgG and titer determination of purified anti-myostatin antibodies ................................................................. 41
2.3.5 Western blot analysis of binding characteristics of polyclonal antibodies ................................................................. 41
2.4. DISCUSSION .................................................................................................................. 57
REFERENCES ....................................................................................................................... 59
APPENDIX ............................................................................................................................ 77
1. Fractionation of total RNA in 1% Agarose gel electrophoresis ...................................... 79
2. Agarose gel (1.2%) electrophoresis of PCR products (1125 bp)
    obtained from RT-PCR using RNAs isolated from both chicken heart and skeletal muscles ................................................................. 80
3. Agarose gel (1.2%) electrophoresis of PCR product (366 bp)
    obtained from RT-PCR using mRNAs isolated from chicken skeletal muscle ................. 81
4. Map of Topo cloning site .................................................................................................. 82
5. PCR identification of positive transformants with correct orientation of inserted fragment (366 bp) ................................................................. 83
6. PCR identification of positive transformants with correct orientation of inserted fragment (1125 bp) ................................................................. 84
7. Titer determination by ELISA, A) anti-mature myostatin
    B) anti-prepro- myostatin ................................................................................................. 85
8. Solubility of 37 kD protein at different pH ....................................................................... 87
9. Effect of centrifugation force on the extraction of 37 kD protein ....................................... 89
10. Examination of the presence of the 37 kD protein in the whole homogenate and supernatant of various tissues from 21 day old chicken embryo ......................... 91

11. Examination of the binding sensitivity of the polyclonal anti-myostatin antibodies at various dilutions in liver and skeletal muscle ......................... 92

12. Examination of the binding of anti-myostatin antibodies in various amount of skeletal muscle ................................................................. 94

13. Posthatch growth after in ovo injection of anti-prepro-myostatin antibody ........... 96
<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>48</td>
</tr>
<tr>
<td>Average value of titer by ELISA with recombinant prepro-myostatin coating antigen</td>
<td></td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Compartmentalization of the somite during vertebrate embryogenesis</td>
</tr>
<tr>
<td>1.2</td>
<td>Interactions between axial and lateral signaling molecules during somatic compartment specification</td>
</tr>
<tr>
<td>1.3</td>
<td>Hierarchical and functional relationships between members of the MRF family</td>
</tr>
<tr>
<td>1.4</td>
<td>Fiber numbers and fiber diameters for the biceps brachii of male and female mice of different ages</td>
</tr>
<tr>
<td>2.1</td>
<td>Predicted amino acid sequence of recombinant chicken myostatin fragments</td>
</tr>
<tr>
<td>2.2</td>
<td>SDS-PAGE analysis of myostatin expression (366 bp)</td>
</tr>
<tr>
<td>2.3</td>
<td>SDS-PAGE analysis of myostatin expression (1125 bp)</td>
</tr>
<tr>
<td>2.4</td>
<td>SDS-PAGE analysis of purified recombinant myostatin used for immunogens</td>
</tr>
<tr>
<td>2.5</td>
<td>SDS-PAGE analysis of affinity purified anti-myostatin IgG</td>
</tr>
</tbody>
</table>
| 2.6    | Titer determination of protein A purified IgGs by ELISA  
A) mature form myostatin as a coating antigen  
B) prepro-myostatin as a coating antigen |
| 2.7    | Western blot analysis of binding characteristics of polyclonal anti-myostatin antibodies to recombinant chicken myostatins, liver and skeletal muscle proteins |
| 2.8    | Western blot analysis of binding characteristics of prepro-myostatin |
antibody in various tissues of chicken ......................... 54

2.9 Western blot analysis of binding characteristics of anti-prepro-myostatin antibody in skeletal muscle from chicken, mice, pig and cattle .... 56

2.10 Western blot analysis of binding characteristics of commercially available polyclonal antibodies generated against C-terminal (GDF-8, C20) and N-terminal (GDF-8, N19) in chicken liver and skeletal muscle homogenates .................... 77
CHAPTER 1
LITERATURE REVIEW

1.1 SKELETAL MUSCLE GROWTH AND DEVELOPMENT

1.1.1 Embryonic development of skeletal muscles

1.1.1.1 Origin of skeletal muscle cells during embryogenesis

Muscle develops from mesoderm, one of the three pluripotent germ layers (endoderm, mesoderm and ectoderm) of early embryo. In addition to muscle, mesoderm also gives rise to the hemopoietic system, cartilage, bone and heart among other tissues. Endoderm gives rise to the digestive and respiratory systems, and ectoderm contributes to the generation of nervous system. Intra-embryonic mesoderm differentiates into paraxial, intermediate and lateral plate mesodermal layers. Among those three mesodermal layers, the paraxial mesoderm contributes to the development of skeletal muscles. The paraxial mesoderm forms into two longitudinal strips of tissue flanking the neural tube, thus separating the neural tube from the intermediate mesoderm, which develops into the ducts and tubules of urogenital system, and also the more laterally positioned lateral plate mesoderm, which develops into the cardiovascular system, lining of body cavities and body wall (Gilbert, 2000).

The unique feature of the paraxial mesoderm is its segmentation into somites, which are the main source of muscle forming cells in the vertebrate embryo (reviewed by Stockdale et al., 2000; Buckingham et al., 2003). Somites are assembled sequentially from head to tail during early embryogenesis. Somites are initially composed of columnar epithelial-like cells arranged radially around small lumen. During maturation, somites differentiate into ventrolateral sclerotome that gives rise to axial skeleton and
ribs and dorsomedial dermomyotome that contributes to the formation of skin and skeletal muscles. Two distinctive compartments present in dermomyotome give rise to separate lineage of skeletal muscles during embryogenesis: dorsomedial epaxial domain gives rise to the deep back and intercostal muscles, and the lateral hypaxial domain gives rise to the rest of the musculature of the body and limbs (Ordahl and Le Douarin, 1992; Miller et al., 1999). The hypaxial dermomyotome gives rise to muscles in two distinct ways: ventrolateral extension of the dermomyotome forms the body wall muscles, and some migratory precursor cells leaving the dermomyotome form more distant muscle masses of the wing and limb (Ordahl and Le Douarin, 1992; Miller et al., 1999). Figure 1.1 illustrates the compartmentalization of somites during vertebrate embryogenesis. While most muscles in vertebrate are originated from somites, some muscles in the head have been shown to be originated from non-somitic paraxial head mesoderm lying rostral to the first somites (Hacker and Guthrie, 1998; Noden et al., 1999).

1.1.1.2 Role of molecular factors in somite development

As discussed in the previous section, medio-lateral polarity exists during the somite development. Switch-graft experiments have shown that during the early somite development the cells in somite are not committed to particular fates in either the mediolateral or the dorsoventral compartments, indicating that the newly formed somites are not yet determined along the mediolateral axis. The determination of medial and lateral somitic compartments occurs after somite formation in response to extrinsic cues provided by the surrounding environments, including the notochord, neural tube and lateral plate (Hirsinger et al., 1998). Molecules including BMP4, Noggin, Wnt and SHH have been identified to be involved in the somatic compartmental specification.
BMP4, as a member of the TGF-β superfamily, is produced by the lateral plate and appears to mediate the lateral plate effect (Figure 1.2). Grafting BMP4-producing cells into the medial somites in chicken embryo was able to make the medial somite cells express lateral somite-specific marker Sim1, indicating a conversion of the medial somites into lateral somites (Pourquie et al., 1996; Tonegawa et al., 1997). Furthermore, BMP4 antagonist Noggin could block the lateral signaling (Hirsinger et al., 1997). Grafting of Noggin expressing cells to the lateral somite in chicken embryo converted the lateral somite to medial somite phenotype as was evidenced by the down-regulation of Sim1 and up-regulation of MyoD, a medial specific molecular marker during the somite development. In addition to the role of lateral somite specification, BMP4 is known to be involved in several inductive events. For example, high levels of BMP4 signaling are required for differentiation of notochord, muscle, pronephros and blood during embryonic development in Xenopus and avian species (Dosch et al., 1997; Tonegawa et al., 1997).

Wntl signaling produced from the neural tube appears to act as a medialising factor on the somite (Figure 1.2). When Wntl expressing cells were grafted to the lateral position in chicken embryo, the lateral compartment displayed properties of the medial compartment of the somites (Hirsinger et al., 1998). This result is similar to the one obtained after neural tube grafting to the lateral compartment (Hirsinger et al., 1998). Studies indicate that the effect of Wntl is mediated by Noggin that is produced mostly from the medial dermomyotome. When the neural tube was removed from the chicken embryo, Noggin expression disappeared, indicating that the Noggin expression in
somites is under the control of the neural tube (Zimmerman et al., 1996; Marcelle et al., 1997; Hirsinger et al., 1998). When the neural tube was replaced by Wnt1 expressing cells in chicken embryo, Noggin expression in the medial position was rescued with the medio-lateral patterning (Hirsinger et al., 1998). The result suggests that the Wnt1 can act like a neural tube factor to regulate Noggin expression. In addition to the Wnt1 signaling generated from the neural tube, SHH produced by the developing notochord activated ectopic expression of Noggin, resulting in the blocking of BMP4 specification of the lateral somite (Hirsinger et al., 1997).

1.1.1.3 Myoblast proliferation and differentiation

During embryonic development, multi-nucleated skeletal muscle fibers are formed from the fusion of single-nucleated myoblasts, which arise mostly from the precursor cells of myogenic lineage in somites. Proliferating myoblasts irreversibly exit from the cell cycle to become fusion competent myoblasts that expresses genes encoding muscle-specific proteins, followed by fusion to form myotubes. Myoblast proliferation and differentiation appears to be mutually exclusive phenomena during muscle development. Environmental signals including growth factors regulate the sustained proliferation of myoblasts and subsequent withdrawal from the cell cycle as part of differentiation pathway. The timing of entry of myoblasts into the differentiation pathway is likely to play a significant role in determining the number of muscle fibers, thus ultimate muscle mass in adult animals.

Transcription factors commonly called myogenic regulatory factors (MRFs) play a crucial role in muscle cell specific commitment and differentiation (Pownall et al., 2002). In addition, various growth factors have been shown to be involved in the
regulation of the myogenic process. Among those growth factors, insulin like growth factor-1 (IGF-1), fibroblast growth factor (FGF) and transforming growth factor (TGF)-β are well characterized for their role in the regulation of myoblast proliferation and differentiation (reviewed by Florini et al., 1996).

1.1.1.3.1 Myogenic regulatory factors

Myogenic regulatory factors (MRFs) form a family of basic-Helix-Loop-Helix (bHLH) transcription factors consisting of Myf5, MyoD, myogenin and MRF4. The MRFs play key roles in the development of skeletal muscle during embryogenesis. MRFs are expressed exclusively in skeletal muscle, and ectopic expression of the MRFs in a wide range of cultured cells induces the skeletal muscle differentiation program. These transcription factors appear to have a master regulatory role in the development of skeletal muscle lineage.

Gene targeting studies using the introduction of null mutations of individual and combinational Myf5, MyoD, myogenin and MRF4 into the germ line of mice revealed a hierarchical relationship among the MRFs and established that functional redundancy is a feature of the MRF regulatory network (Megeney and Rudnicki, 1995). Figure 1.3 briefly summarizes the results of the various gene targeting studies. The MRF family can be divided into two functional groups; the primary MRFs (MyoD and Myf5) are required for the determination of skeletal myoblasts, and the secondary MRFs (myogenin and MRF4) act later in the program as differentiation factors. The primary MRFs are expressed in proliferating myoblasts before differentiation, and the secondary MRFs are expressed in terminally differentiating cells.

Mice lacking functional MyoD gene were found to have no obvious
abnormalities in skeletal muscle but expressed four-fold higher level of Myf5, suggesting
the functional redundancy between MyoD and Myf5 (Rudnicki et al., 1992). Newborn
mice lacking Myf5 also had normal skeletal muscle (Braun et al., 1992). Newborn
mice lacking both MyoD and Myf5 have a complete absence of skeletal muscle
(Rudnicki et al., 1993). While there is a functional redundancy between MyoD and
Myf5, each MRF appears to have their own unique functions. For example, newborn
Myf5-deficient animals display normal skeletal muscle development but die prenatally
because of severe rib abnormalities, indicating the requirement of Myf5 for normal rib
development (Braun et al., 1992).

Once myogenic cells have been specified, skeletal muscle development requires
differentiation and fusion of myoblasts to form multinucleated myotubes and myofibers.
Myogenin has shown to play an important role in the differentiation of myoblast into
myotube and myofibers. Mice lacking a functional myogenin gene die at birth due to the
lack of myofibers (Hasty et al., 1993; Nabeshima et al., 1993). However, normal
numbers of MyoD-expressing myoblast are present in the myogenin lacking mice.
MRF4 mutation displays normal Myf-5 expression and a four-fold induction in
myogenin, and is viable with seemingly normal skeletal muscle (Zhang et al., 1995).
Mice lacking both Myf-5 and MRF4 closely resemble mice lacking only Myf-5. Thus,
MRF4 may function late in the myogenic pathway, and its function may be substituted
by the presence of myogenin (Braun and Arnold, 1995).

In addition to the MRFs, the myocyte enhance factor-2 (MEF2) family of
transcription factors is involved in the activation of muscle specific gene expression in
mice (Naya and Olson, 1999) and in Drosophila (Baylies and Michelson, 2001). MEF2
proteins are expressed in many tissues but it is only in the developing cardiac, skeletal and smooth muscles that MEF2 activates transcription (Naya and Olson, 1999). The MEF2 family and the MRFs function synergetically to activate skeletal muscle specific promoters and activate each other’s expression. The onset of differentiation, specifically gene expression in skeletal muscle, is coupled to permanent withdrawal from the cell cycle. The retioblastoma tumor (pRb) suppressor protein is critical regulator of this process, required for cell cycle arrest in G0 phase. Its high level expression during the late muscle development makes it a good marker for the late muscle development (Novitch et al., 1996 and 1999). The myogenic defect observed in Rb^- cells appears to be caused by a deficiency in the activity of the transcription factor MEF2. Without pRb, MyoD induces the accumulation of nuclear localized MEF2 that is competent to bind DNA yet transcriptionally inert (Novitch et al., 1999). When pRb is present, MyoD stimulates the function of the MEF2 transcriptional activation domain and the activity of endogenous MEF2 factors (Novitch et al., 1999). This indicates that Rb promotes the expression of late stage muscle differentiation markers by both inhibiting cell cycle progression and cooperating with MyoD to promote the transcriptional activity of MEF2 (Novitch et al., 1996 and 1999).

1.1.1.3.2 Signals regulating MRF expression

Studies have demonstrated that signals from structures surrounding somites are involved in the regulation of MRFs expression. Signals from the neural tube and notochord activate epaxial myogenesis in the dorsal medial domain of the somite (Chiang et al., 1996). For example, the absence of the notochord, or a defect in neural tube and notochord differentiation prevented epaxial myogenesis in mouse embryos
The notochord and the floor plate produce sonic hedgehog (SHH) which is required for the expression of Myf5 in epaxial muscle precursors. SHH was unable to activate MyoD transcription in Myf5-/- somites, which indicates that SHH induces myogenesis specifically through Myf5 (Borycki et al., 1999). SHH signaling activates the expression of Myf5 directly through the Gli family of transcriptional regulators, the specific binding site of which has been identified in the Myf5 epaxial somite enhancer. However, the Myf5 expression in the epaxial domain appears not to be completely dependent upon SHH signaling because SHH null mice showed that Myf5 expression was reduced but not absent in the epaxial domain (Kruger et al., 2001). The SHH null mice have also lost hypaxial musculature, indicating that hypaxial muscle formation might depend on Myf5 expression at least in part. Conversely, retrovirally overexpressed SHH in the limb bud in vivo induced the over expression of MyoD gene and finally that of the myosin protein. This led to hypertrophy of the muscles in vivo. SHH added to primary cultures of myoblasts resulted in an increase in the proportion of myoblast that incorporate bromodeoxyuridine, resulting in an increase of myotube number (Duprez et al., 1998). These studies showed that SHH was able to activate myogenesis in vivo and in vitro in already committed myoblast, and suggested that the stimulation of the myogenic program by SHH involved activation of cell proliferation.

Signals from the lateral plate mesoderm and the dorsal ectoderm appear to combine to regulate hypaxial muscle formation. The dorsal ectoderm provides signals such as Wnts that stimulate myogenesis and activate the expression of MyoD, whereas molecules such as BMPs, Notch signaling and Msx1 that are derived from the lateral
plate mesoderm inhibit myogenesis (Pourquie et al., 1995 and 1996; Cossu et al., 1996). Signals of Wnt1 and Wnt7a are expressed in dorsal neural tube and dorsal ectoderm, respectively. SHH synergises with both Wnt1 and Wnt7a in explants of mouse from E8.5 epaxial mesoderm (Tajbakhsh et al., 1998). Specifically, Wnt7a activates the expression of MyoD in explants of paraxial mesoderm, which in turn leads to the co-expression of both MyoD and Myf5. Soluble Frizzled-related proteins (sFRPs), which specifically block Wnt signaling, inhibit myogenesis in explanted somitic mesoderm or newly formed somites, supporting the positive role of Wnt in myogenesis. The inhibition of myogenesis by sFRPs is accompanied by the downregulation of Noggin and Myf5 with no effect on the expression of either Pax3 or mesenchyme homeobox-1 (Parker et al., 2003). Because Pax3 is involved in the early lineage specification, the result indicates that the disruption of Wnt signaling by sFRPs might not alter early aspects of lineage specification such as Pax3 expression, but disrupts myogenesis by blocking the expression of MyoD and Myf5.

BMPs, especially BMP2, 4 and 7, can replace the inhibitory activity of lateral-plate mesoderm on activation of MyoD, yet maintain Pax3 expression in the somites (Pourquie et al., 1996; Amthor et al., 1998; Dietrich et al., 1998). This result indicates that BMPs might function to establish a sufficient number of myogenic progenitors before terminal differentiation. Furthermore, Noggin, a secreted BMP antagonist, is expressed in the dorsal medial lip under the control of Wnt signaling and facilitates the expression of MyoD (Hirsinger et al., 1997). The exposure of developing somites to excess Noggin induced premature differentiation and prevents sufficient myoblast expansion (Reshef et al., 1998). Moreover, Noggin\(^{−/−}\) mice has a severe reduction in the
differentiation of the epaxial myotome (McMahon et al., 1998).

The Notch signaling pathway appears to acts as lateral inhibition of muscle differentiation through the activation of MyoD expression. The overexpression of the Notch ligand Delta-1 in chicken somites inhibited the expression of MyoD in the myotome and blocked differentiation, yet expressions of Pax3 and Myf5 were maintained, and a myotomal structure was formed (Hirsinger et al., 2001). Also, overexpression of Delta-1 in the chicken limb inhibited muscle differentiation without affecting Myf5 and Pax3 expression (Delfini et al., 2000).

Among various transcription factors expressed during myogenesis, Pax3 expression is known to be essential for migration of myogenic precursor cells, a process required for limb musculature formation (Goulding et al., 1994). The expression of Pax3 in the presomitic mesoderm and early epithelial somites marks the early stage of commitment of skeletal muscle cell lineage (Goulding et al., 1994). In addition, Pax3 has shown to play a broader role in skeletal muscle differentiation, such as activation of myogenic regulatory factors (Bendall et al., 1999; Maroto et al., 1997). In contrast to Pax3, the homeobox transcription factor Msx1 that is expressed in the lateral domain of the dermomyotome and migrating limb muscle precursor cells appears to inhibit the muscle differentiation. Ectopic expression of Msx1 downregulated the expression of MyoD and inhibited myoblast differentiation without altering Pax3 expression in chicken embryos (Bendall et al., 1999). Since ectopic expression of Pax3 induced the expression of MyoD (Bendall et al., 1999), it appears that Msx1 and Pax3 have opposite effect on the expression of MyoD. The above study, furthermore, demonstrated that the Msx1 protein inhibits the DNA binding of Pax3. Therefore, it was proposed that the
suppression of myogenic activity in migrating limb muscle precursor cells is caused by the antagonizing effect of Msx1 on Pax3 via direct protein-protein interaction (Bendall et al., 1999).

1.1.2 Postnatal skeletal muscle growth

1.1.2.1. Muscle fiber number

Studies have shown that the number of skeletal muscle fibers in most mammals and birds does not increase after the embryonic proliferation of skeletal muscle has been completed (Goldspink, 1962; Rowe and Goldspink, 1969). While muscle cell proliferation is most active during embryonic development, limited extent of muscle cell proliferation appears to occur after birth in some species since increase in the number of muscle fibers during the neonatal period has been observed (Rayne and Crawford, 1975; Swatland, 1976; Wigmore and Stickland, 1983). The postnatal increase in skeletal muscle mass is, therefore, achieved mostly by an increase in fiber size and not much by an increase in fiber numbers (Figure 1.4). Muscle fiber in young animals has a smaller cross sectional area than that in older animals and contains less nuclei with a smaller volume. Since fiber size cannot be increased beyond a certain limit, the growth potential for skeletal muscle is virtually determined by the number of fibers established at around the time of birth. This relationship of muscle number and growth potential has been demonstrated in the enlarged muscles of double-muscled cattle (Swatland and Kieffer, 1974), in genetically different size of animals (Hanrahan et al., 1973), and in runts as compared with normal pigs (Hegarty and Allen, 1978; Powell and Aberle, 1981).

1.1.2.2. Satellite cells
Muscle mass increases more than 10 fold from birth to mature body size, but DNA: protein ratio decreases slightly during this period, indicating an increase in the number of myonuclei during the postnatal hypertrophy. During the postnatal enlargement of muscle fiber, mitotically active satellite cells provide myonuclei to the enlarging fibers to balance the cytoplasm/nuclei ratio (Moss and Leblond, 1971). Satellite cells are mononucleated cells that are located between the basal lamina and sarcolemma of myofibers. Unlike the nuclei inside muscle fibers, the satellites cells retain the capacity to proliferate in response to stimuli, thus provide most of the myonuclei to adult muscle (Schultz, 1996).

The MRF expression program during satellite cell activation, proliferation and differentiation is analogous to the program manifested during the embryonic development of skeletal muscle. Quiescent satellite cells express no detectable levels of MRFs. In mouse skeletal muscles, activated satellite cells (satellite cells entering the cell cycle) first express either Myf5 or MyoD followed soon after by co-expression of Myf5 and MyoD (Seal and Rudnicki, 2000). Following proliferation, myogenin and MRF4 are expressed in cells beginning their differentiation program (Cornelison and Wold, 1997).

1.2 MYOSTATIN, A NEGATIVE REGULATOR OF SKELETAL MUSCLE GROWTH

Myostatin is a new member of the TGF-β superfamily of proteins (McPherron et al., 1997). The disruption of myostatin expression by gene targeting resulted in 2-3 times bigger muscle mass as compared to the wild type in mice (McPherron et al., 1997).
The increase in muscle mass was due to both muscle cell hyperplasia and hypertrophy. The Belgian Blue and Piedmontese breeds of cattle, which are characterized by an increase in muscle mass (double muscling), have been found to have a mutation in the myostatin gene (Grobet et al., 1997; Kambadur et al., 1997; McPherron and Lee, 1997). Similarly, a “compact” mutation in mouse, which is characterized by an increase in protein content in the carcass, was found to be caused by a mutation in the myostatin gene (Szabo et al., 1998). These results indicate that myostatin negatively regulates skeletal muscle growth.

Myostatin was initially reported to be (McPherron et al., 1997) expressed in skeletal muscle, but recent studies suggest an expression in other tissues. Myostatin mRNA was expressed in both myogenic cells (myoblast) and non-myogenic cells (fibroblast) in regenerating skeletal muscle of the rat (Yamanouchi et al., 2000). Myostatin mRNA was detected in Perkinje fibers and cardiomyocytes in heart tissue (Sharma et al., 1999). Ji et al. (1998) reported an expression of myostatin mRNA in tubuloalveolar secretory lobules of lactating mammary gland, suggesting that myostatin might be involved in the regulation of gestational or lactational mammary gland growth and development and/or metabolism.

1.2.1 Biochemical characteristics of myostatin

Like many other TGF-β family members proteins, myostatin appears to be produced as a precursor protein composed of a signal sequence, an N-terminal propeptide domain (prodomain) and a C-terminal active domain (Zimmers et al., 2002). Based on cDNA sequence, the precursor form of myostatin is known to comprise 375
amino acid (AA) in humans, baboons, cattle, pigs, sheep, turkeys and chickens, and 376 AA in rodents (McPherron and Lee, 1997). The mature or active form of myostatin consisting 109 AA appears to be formed upon removal of the N-terminal prodomain (also called latent peptide) by proteolysis at the tetrabasic (RSRR) site (Lee and McPherron, 2001). The mature form of myostatin has identical AA among human, murine, rat, procine, chicken and turkey species, and 1 AA difference in baboon, 2 AA differences in bovine and 3 AA difference in ovine species (McPherron and Lee, 1997).

The mature form of myostatin as well as the precursor form of myostatin appears to form a disulfide-linked dimer like many other members of the TGF-β superfamily (McPherron et al., 1997; Lee and McPherron, 2001). In a western blot analysis of proteins from Chinese hamster ovary (CHO) cell culture carrying copies of a murine myostatin expression construct, two myostatin-immunoreactive proteins were detected at 52 kD and 15 kD under reducing conditions, representing probably unprocessed precursor and mature form of myostatin, respectively (McPherron et al., 1997). Under non-reducing conditions, two proteins of 101 kD and 25 kD were detected, consistent with dimeric forms of precursor and mature myostatin, respectively. The dimer formation of the mature form of myostatin was also demonstrated in in vitro system in other studies (Wehling et al., 2000; Lee and McPherron, 2001; Thies et al., 2001). Recent studies reported that the apparent molecular weight of myostatin prodomain in SDS-PAGE was 37 kD (Lee and McPherron, 2001; Thies et al., 2001). However, the predicted molecular weight of prodomain would be 27 kD based on the known amino acid sequence. Since myostatin prodomain (37 kD) was retained in a lentil lectin sepharose column, it was suggested that the discrepancy in estimated and
apparent molecular weight was due to a glycosylation of the myostatin prodomain (Lee and McPherron, 2001), a common characteristic of the TGF-β superfamily member proteins. The myostatin prodomain did not produce disulfide-linked dimer formation (Lee and McPherron, 2001; Thies et al., 2001).

Many studies reported that myostatin-immunoreactive proteins in skeletal muscle and serum (Sharma et al., 1999; Berry et al., 2002, Wehling et al., 2000; Sakuma et al., 2000; Lalani et al., 2000; Kawada et al., 2001), but the apparent molecular weight and reversible disulfide-linkage formation of the myostatin-immunoreactive protein in SDS-PAGE have been inconsistent. Therefore, the post-translational processing of myostatin is yet to be established in in vivo animal systems. In bovine skeletal muscles, three myostatin-immunoreactive proteins were identified at 52 kD, 40 kD and 26 kD ~ 30 kD (Sharma et al., 1999; Berry et al., 2002). In human skeletal muscles, a 26 kD myostatin-immunoreactive protein was detected under both reducing and non-reducing conditions (Gonzalez-Cadavid et al., 1998). The 26 kD band was also detectable in serum. Because the 26 kD band had an affinity to ConA-Sepharose column, they suggested that this presumable mature form of myostatin was glycosylated. In rat skeletal muscles, myostatin-immunoreactive protein was detected as a 30 kD or 32 kD band under both reducing and non-reducing conditions (Lalani et al., 2000; Sakuma et al., 2000; Kawada et al., 2001). In C2C12 muscle cell culture, myostatin-immunoreactive protein was also detected as a 30 kD band in the nuclear fraction (Artaza et al., 2002).

Recently, two myostatin-immunoreactive proteins were separated 12 kD and 36 kD from mouse serum using immuno-precipitation, and using LC-MS-MS methods, the 12 kD and 36 kD protein were identified as mature and prodomain myostatin,
respectively (Hill et al., 2002). The presence of the 12 kD mature myostatin was also reported in mouse serum (Zimmers et al., 2002). Therefore, the two recent results strongly suggest that some of the myostatin-immunoreactive proteins discussed above were probably a non-myostatin protein that had affinity to anti-myostatin antibodies.

1.2.2 Genetics of myostatin

Myostatin gene was mapped to a region in chromosome 2 (BTA2) at q11 in cattle, where the double muscling (muscular hypertrophy; mh) locus had been located (Sonstegard et al., 1997; Grobet et al., 1998). In human, it was mapped to chromosome 2q (HSA2q) at q32·1 that is syntenic to the mh locus of double muscled cattle (McPherron and Lee, 1997; Gonzalez-Cadavid et al., 1998). In pigs (Sonstegard et al., 1998), mice (McPherron et al., 1997) and chicken (Sazanov et al., 1999), it was mapped to chromosome 15 (15q2·3), chromosome 1 and chromosome 7 (GGA 7p11), respectively.

The human myostatin gene comprises three exons and two introns and has three transcription initiation sites, with no introns being present in the 5′-untranslated region upstream of the initiation codon (Gonzalez-Cadavid et al., 1998). The general genomic organization appears to be similar to that of other members of transforming growth factor-β family (Guron et al., 1995). The pig and cattle myostatin genes were also reported to comprise three exon and two introns (Stratil and Kopecny, 1999). The human myostatin gene promoter contains two upstream TATA boxes, AP-1 transcription factor binding site, MyoD responsive element (Ferrell et al., 1999). They also observed that human myostatin gene contained at least 5 SNPs (single-nucleotide polymorphisms)
site in exons 1 and 2, but no SNP site in exon 3. According to a recent analyses of 3.3-kb human myostatin promoter region, it contained three TATA boxes, a partial CCAAT box, five octameric sequences homologous to the consensus binding sites of POU homeodomain proteins, twelve E boxes corresponding to MyoD binding sites, two regions homologous to MEF2 binding site, a putative peroxisome proliferators-activated receptor-γ (PPAR-γ) binding site and a region homologous to the consensus sequence of the nuclear factor (NF)-κB binding site (Ma et al., 2001). Also, identified were consensus sequences of various hormone binding sites including androgen response element, five sequences corresponding to three different glucocorticoid response elements [GRE, palindromic GRE (pal-GRE), and tyrosine aminotransferase GRE (tat-GRE)], three thyroid hormone response element (TRE), three ER6 sequences that are known to have similar function to TRE and two regions homologous to a cAMP response element. In support of the stimulatory role of glucocorticoid on myostatin expression, the administration of dexamethasone, a glucocorticoid agonist, upregulated myostatin mRNA expression in rat skeletal muscles (Lang et al., 2001) and in muscle cell culture models (Ma et al., 2001). The increase in myostatin mRNA by dexamethasone was blocked by the administration of glucocorticoid antagonist, RU486, in both in vivo and in vitro. The functional significance of the other sequence elements on the regulation of myostatin expression is yet to be determined.

1.2.3 Physiological action of myostatin

1.2.3.1 Role of myostatin in the control of myoblast proliferation and differentiation

While most growth factors play a role in a wide variety of cell type, the role of
myostatin appears to be mostly confined to skeletal muscles as was evidenced by the
dramatic effect of myostatin knockout on skeletal muscle mass without much impact on
other organs (McPherron et al., 1997). In skeletal muscle cell cultures, addition of
recombinant myostatin or over-expression of myosatin inhibited the proliferation of
myoblasts (Thomas et al., 2000; Taylor et al., 2001). Levels of myostatin mRNA
expression during chicken embryonic development and in muscle cell culture support the
inhibitory role of myostatin on myoblast proliferation because the lower level of
myostatin mRNA coincides generally with the period of myoblast proliferation before
myotube formation (Kocamis et al., 1999 and 2001). The inhibitory role of myostatin
on myoblast proliferation explains the hyperplasia of muscle fibers observed in mice and
cattle carrying a non-functional myostatin gene. In the pig, myostatin mRNA was
detectable in whole fetuses at 21 and 35 days of gestation and was markedly increased by
49 days (Ji et al., 1998). At birth, mRNA abundance in longissimus muscle had
decreased significantly from the level at day 105 of gestation and continued to decrease to
its lowest level at 2 weeks postnatally (Ji et al., 1998). The reduction in myostatin
mRNA abundance at birth and postnatal period coincides also with the period of
reduction in myoblast mitogenic activity and differentiation.

The inhibition of the proliferation of myoblasts by myostatin appears to be
through the prevention of the progression of myoblasts from the G1-phase to S-phase of
the cell cycle regulated by Rb protein hypophosphorylation (Thomas et al., 2000; Joulia
et al., 2003). The maintenance of the proliferative capacity of myoblasts after removing
myostatin from the cell culture indicated that the inhibitory effect of myostatin was
reversible (Taylor et al., 2001). Similarly, upregulation of cyclin dependent kinase
inhibitor p21 and down regulation of cyclin dependent kinase (Cdk2) were observed when myostatin was added in muscle cell culture (Thomas et al., 2000). The upregulation of p21 was also observed in C2C12 muscle cell cultures over-expressing myostatin (Rios et al., 2001). Because Cds are the key regulatory molecules for cell proliferation, these results indicate that myostatin inhibit myoblast proliferation through the Cdk system. The myostatin mRNA levels in two different in vitro myogenic differentiation models such as in mouse BC3H1 and C2C12 cells increased during in vitro differentiation (Mendler et al., 2000). Not only the proliferation but also the differentiation of myoblasts was inhibited by myostatin in a dose dependent manner (Langley et al., 2002). In that study and others (Rios et al., 2002), down regulation of MyoD and other MRFs were observed in myostatin-treated myoblasts. Smad3 is a down stream mediator of TGF-β receptors, thus the Smad3 expression was monitored during the myoblast differentiation in cell culture system treated with myostatin (Langley et al., 2002). The level of phosphorylated Smad3 was up-regulated by the addition of myostatin in the culture (Langley et al., 2002). Similarly, when cultured C2C12 cells were treated with blocking antibodies generated against myostatin, cells treated with myostatin antibody had decreased concentrations of phosphorylated Smad2 and 3 compared with controls (Bogdanovich et al., 2002).

1.2.3.2 Role of myostatin in the control of post-natal muscle growth

In addition to the inhibitory role of myostatin in myoblast proliferation, numerous studies indicate that myostatin has a role in skeletal muscle growth and maintenance in postnatal animals. In a study by Carlson et al. (1999), myostatin mRNA abundance was examined in mice gastrocnemius-plantaris muscles undergoing hindlimb
unloading at 1, 3 and 7 days. Significant muscle atrophy was observed at 3 day after unloading. However, myostatin mRNA increased significantly in mice gastrocnemius and plantaris muscles at 1 day after hindlimb unloading but not at 3 or 7 days of hindlimb unloading, suggesting that the myostatin upregulation probably induced the atrophy of skeletal muscles. Wheling et al. (2000) also reported that 10 days of unloading caused a 16% decrease in plantaris mass and a 110% increase in myostatin mRNA. Consistent with the above results, the expression of myostatin mRNA level increased 30-fold in both chronic and acute disuse-induced muscle atrophy (Reardon et al., 2001). Lalani et al. (2000) investigated whether the muscle loss associated with spaceflight is accompanied by increased levels of myostatin mRNA in skeletal muscles. Myostatin mRNA concentrations were significantly higher in skeletal muscles from the spaceflight rat than those in the ground-based controls, resulting in negative relationship between the myostatin mRNA expression and muscle mass.

The expression of myostatin mRNA was also measured in muscle regeneration models to examine whether myostatin play a role during muscle regeneration. In rat skeletal muscles undergoing necrosis induced by injecting bupivacaine or hypertonic saline solution, myostatin mRNA was expressed in myogenic mononucleated cells during regeneration (Yamanouchi et al., 2000). The results of this study demonstrate that myostatin mRNA expression is induced in regenerating area where activated satellite cells are proliferating. In a study where the temporal expression of mRNA for myostatin was investigated in satellite cell culture derived from chicken muscles, myostatin mRNA level started to increase when fusion started after plating the cells, then myostatin mRNA plateaued through 144 hours after reaching the highest level at 72
hours (Kocamis et al., 2001). This result suggests that myostatin mRNA probably functions as an inhibitor of satellite cell proliferation. The spatial and temporal expression of myostatin mRNA was investigated during regeneration after cardiotoxin injury in mouse soleus muscle (Armand et al., 2003). Myostatin mRNA levels declined gradually during degeneration caused by notexin injection and returned close to the normal level at the completion of regeneration at 28 days after the notexin injection in rat soleus and extensor digitorum longus muscles in vivo (Mendler et al., 2000). The in vivo regenerating rat skeletal muscle demonstrated that myostatin expression is suppressed during the early stage of regeneration when satellite cell derived myoblasts proliferate, then the expression of myostatin mRNA returns to normal levels after the completion of regeneration.

Some studies suggest that the extent of expression of myostatin mRNA is dependent upon muscle types. The expression of myostatin mRNA was higher in fast type muscle than in slow type muscle (Mendler et al., 2000). Similarly, the greatest amount of myostatin mRNA was found in the white portion of mouse quadriceps muscle that was composed of 100% type IIB (Carlson et al., 1999). While it is not clear whether myostatin plays a role in the regulation of the abundance of satellite cells, animal studies have shown that predominantly white fiber has low satellite cell densities compared to muscles that have predominantly red fibers (Carlson et al., 1999; Wehling et al., 2000).

More strong evidence supporting the negative role of myostatin in postnatal skeletal muscle growth and maintenance come from studies using anti-myostatin antibodies as a vehicle to suppress the biological activity of myostatin. Bogdanovich et
al. (2002) produced mouse anti-myostatin monoclonal antibody which was generated against recombinant myostatin and administrated the anti-myostatin antibodies to mdx mice (an animal model for Duchenne muscular dystrophy). These mice injected with the anti-myostatin antibody gained weight faster than controls, thus weighed significantly more than controls after 3 months of treatment. Increases in whole muscle cross sectional area and muscle fiber area were also observed (Bogdanovich et al., 2002). Whittemore et al. (2003) produced monoclonal anti-myostatin antibody by immunizing myostatin knockout mice with recombinant myostatin purified from myostatin expressing CHO cell. When they administered the antibody to mice, the antibody-treated mice gained approximately 10% more weight than the control. The mass of the muscle for the antibody-treated mice was greater than the control mice. The negative role of myostatin in postnatal skeletal muscle growth is also found in studies using transgenic animal models. Since myostatin prodomain binds to mature myostatin and inhibits myostatin biological activity, Yang et al. (2001) generated transgenic mice overexpressing myostatin prodomain postnatally in an effort to inhibit biological activity of myostatin postnatally. The transgenic mice overexpressing prodomain exhibited an increase in skeletal muscle mass, and the increase in muscle mass was due to hypertrophy of muscle fiber. More recently, Grobet et al. (2003) generated conditional myostatin knockout mice that demonstrated postnatal inactivation of myostatin. They reported that the conditional myostatin knockout mice had significantly increased muscle mass as was demonstrated in the constitutive myostatin knockout mice generated by McPherron et al. (1997). However, unlike the constitutive myostatin knockout mice, they reported that the increase in skeletal muscle mass in the conditional myostatin
knockout mice was primarily due to the hypertrophy of skeletal muscle fibers. In contrast to the inhibition of biological activity of myostatin by anti-myostatin antibody, over-expression of myostatin in mice induced a decrease in muscle mass similar to the human cachexia condition (Zimmers at al., 2002), indicating an inhibitory role of myostatin on postnatal skeletal muscle growth. These results suggest that the adult skeletal muscle mass can be increased by inhibiting myostatin activity.

1.2.3.3 Role of myostatin on adipose tissue metabolism

McPherron and Lee (2002) reported that myostatin null mice had a significant reduction in fat accumulation with increasing age compared with wild type mice without changes in food intake and body temperature. Conversely, transgenic mice overexpressing myostatin showed an increase fat mass as compared to wild type (Reisz-Porszasz et al., 2003). Interestingly, only the male transgenic mice demonstrated an increase in fat mass but not females in the above study. Lin et al. (2002) examined the expression of peroxisome proliferators-activated receptor-γ (PPAR-γ) and CCAAT/enhancer binding protein –α (C/EBP-α), two major transcription factors involved in adipogenesis, in myostatin knockout mice. Expression level of the two transcription factors in adipose tissue was significantly lower in myostatin knockout mice than in wild type mice. Combining together, the results suggests that myostatin has a positive role in adipogenesis probably through up-regulation of PPAR-γ and C/EBP-α, two key transcription factors involved in adipogenesis.

In contrast to the above results, studies with adipocyte culture indicated that myostatin inhibited the differentiation of preadipocyte into adipocyte (Kim et al., 2001). In this study, recombinant myostatin was added to the 3T3-L1 culture, and expression of
transcription factors involved in adipocyte differentiation was examined between the treated and control cultures. The expression of PPAR-γ and C/EBP-α in treated culture was significantly decreased as compared to the control culture, suggesting myostatin’s inhibition of adipocyte differentiation. In support for the inhibition of differentiation, the morphologic pattern of differentiation was significantly reduced in the myostatin-treated culture. Further studies, therefore, are warranted to understand the exact role of myostatin on adipogenesis and adipose tissue metabolism.

1.2.4. Regulation of myostatin signaling

It is well established that the biological activities of some members of TGF-β superfamily proteins such as TGF- β1, β2, β3 are down regulated by noncovalent association of the prodomain protein with the mature TGF- β1, β2, β3 cleaved from the N-terminal precursor proteins (Miyazono et al., 1988; Wakefield et al., 1988; Brown et al., 1990). Recent studies have indicated that the regulation of biological activity of myostatin is probably very similar to the above process (McPherron et al., 1997; Thies et al., 2001; Lee and McPherron et al., 2001). In support of the inhibitory role of prodomain on the biological activity of myostatin, transgenic mice overexpressing myostatin prodomain had been shown to have a significantly increased muscle mass that wild type mice (Yang et al., 2001). Thies et al. (2001) demonstrated that L6 myoblast cells bound myostatin with high affinity, and myostatin prodomain inhibited the binding of myostatin to L6 cells. Lee and McPherron (2001) has demonstrated that myostatin binds to ActRIIB (active type IIB receptor) in a specific and saturable manner. Furthermore, transgenic mice overexpressing a dominant-negative form of ActRIIB had
increased muscle growth similar to the myostatin null mice, suggesting an involvement
of ActRIIB in myostatin signaling (Lee and McPherron, 2001). In the same study, it
was also demonstrated that the binding of myostatin to ActRIIB was inhibited by
prodomain of myostatin and follistatin that is known to block the activity of several
TGF-β member proteins. Transgenic mice overexpressing follistatin had a significant
increase in muscle mass, indicating an antagonistic role of follistatin for myostatin
activity (Lee and McPherron, 2001). In addition, follistatin could inhibit myostatin
activity in a transcription-based reporter assay (Zimmers et al., 2002).

Others have found that myostatin in serum bind to FLRG (follistatin related
gene), a follistatin domain containing protein that negatively regulates myostatin activity
(Hill et al., 2002). More recently, a novel member of the follistatin domain protein
family that is called growth and differentiation factor (GDF) - associated serum protein-
1(GASP-1) was found to interact with myostatin (Hill et al., 2003). In a reporter gene
assay, the GASP-1 was able to inhibit myostatin activity, suggesting that GASP-1 acts as
a negative regulator of myostatin action. Therefore, it appears that many proteins
including follistatin, FLRG and GASP-1 are interacting with myostatin to regulate the
biological activity of myostatin, and the physiological significance of the interaction of
those proteins with myostatin remains to be determined.
Figure 1.1. Compartmentalization of the somite during vertebrate embryogenesis

From Hawke and Garry, J. Appl. Physiol. 91: 534-551, 2001
Figure 1.2. Interactions between axial and lateral signaling molecules during somatic compartment specification

From Hirsinger et al., Development 124:4605-4617, 1997
Figure 1.3. Hierarchical and functional relationships between members of the MRF family

Figure 1.4. Fiber numbers and fiber diameters for the biceps brachii of male and female mice at different ages (Rowe and Goldspink, 1969). ◆, fiber number; ▣, fiber diameter
CHAPTER 2
PRODUCTION AND CHARACTERIZATION OF POLYCLONAL ANTIBODIES AGAINST CHICKEN MYOSTATINS

2.1 INTRODUCTION

Myostatin, a member of the TGF-β family of growth factors, is a negative regulator of skeletal muscle growth. Myostatin knock out caused a significant increase in muscle mass through muscle cell hypertrophy and hyperplasia (McPherron et al., 1997). Non-functional mutation of myostatin gene was reported in double-muscled cattle that are characterized by a greater muscle mass than normal cattle (McPherron and Lee, 1997; Grobet et al., 1997; Kambadur et al., 1997). Like many other TGF-β family members, myostatin appears to be produced as a prepropeptide composed of a signal sequence, an N-terminal propeptide domain and a C-terminal active domain (Zimmers et al., 2002). The mature form of myostatin is formed upon removal of N-terminal prodomain (also called latent peptide) by proteolysis at the tetrabasic (RSRR) site. Studies have indicated that the binding of prodomain to mature peptide plays a physiological role to inhibit biological activity of myostatin (Lee and McPherron, 2001; Thies et al., 2001; Yang et al., 2001).

Myostatin is mostly expressed in skeletal muscle (McPherron et al., 1997; Kambadur et al., 1997). In skeletal muscle cell cultures, addition of recombinant myostatin or overexpression of myostatin inhibited the proliferation of myoblasts (Thomas et al., 2000; Taylor et al., 2001). Increased levels of myostatin mRNA expression were observed at periods when the myoblast proliferation decreases during
chicken embryonic development (Kocamis et al., 1999 and 2000). Studies with regenerating skeletal muscle model have shown that myostatin mRNA level decrease initially during regeneration but eventually increase to normal level at the later stage of regeneration, when satellite cells cease to proliferate to form myofibers by fusing (Mendler et al., 2000; Yamanouch et al., 2000; Kocamis et al., 2001; Armend et al., 2003). These results indicate that myostatin regulates the proliferation of myoblast during embryonic development and satellite cell proliferation in postnatal muscles.

In addition to the regulation of proliferation of myoblast and satellite cells, myostatin also regulates the hypertrophy of muscle fiber in postnatal animals, as was demonstrated by the negative relationship between myostatin mRNA expression and changes in muscle mass in various physiological conditions including HIV-infected men (Gonzalez-Cadavid et al., 1998), hindlimb unloading (Wehling et al., 2000; Carson et al., 1999), space flight (Lalani et al., 2000) and chronic disuse muscle atrophy (Reardon et al., 2001). In support of the role of myostatin in postnatal skeletal muscle growth, postnatal administration of anti-myostatin antibodies increased muscle mass in mice (Bogdanovich et al., 2002; Whittemore et al., 2003). More recently, Grobet et al. (2003) generated conditional myostatin knockout mice that demonstrated a postnatal inactivation of myostatin. They reported that the conditional myostatin knockout had significantly increased muscle mass as was demonstrated in the constitutive myostatin knockout mice generated by McPherron et al. (1997). However, unlike the constitutive myostatin knockout mice, they reported that the increase in skeletal muscle mass in the conditional myostatin knockout was primarily due to the hypertrophy of skeletal muscle fibers. These findings thus imply that postnatal skeletal muscle growth can be
enhanced by pharmacological agents that block myostatin activity.

Additionally, studies suggest that myostatin is probably involved in the regulation of adipocyte differentiation and metabolism. Myostatin knockout mice had significant reduction in fat accumulation (McPherron and Lee, 2002) and transgenic mice overexpressing myostatin had increased fat mass (Reisz-Porszasz et al., 2003). Studies by Lin et al. (2002) demonstrated that myostatin's stimulatory effect of adipocyte development is probably through positive regulation on the expression of PPARγ and C/EBPα. These findings imply the possibility that pharmacological agents that block myostatin function may be useful not only in enhancing muscle growth in animals and human, but also in slowing or preventing the development of obesity and type II diabetes. Therefore, the objective of this study was to produce and to characterize polyclonal antibodies against chicken myostatins in an effort to use the anti-myostatin antibodies as a vehicle to inhibit the biological activity of myostatin.
2.2 MATERIALS AND METHODS

2.1.1 Amplification of chicken myostatin cDNAs

Total RNA from 5 week-old broiler chicken *pectoralis* muscle was extracted using a commercially available RNA extraction kit (Trizol, Gibco BRL Inc., Rockville, MD). Appendix 1 shows the fractionation of total RNA in 1% agarose gel. The mRNAs were purified from the total RNA using an oligo-dT column. The mRNAs (100 ng) were subjected to oligo-dT primed reverse transcription using 200 U of SuperScript II RNase H- reverse transcriptase (Gibco BRL Inc., Rockville, MD) to synthesize cDNAs. Five μl of the above reverse transcription reaction mixture were used to amplify an 1125 bp unprocessed form of and a 369 bp C-terminal fragment of chicken myostatin cDNA in separate reactions. The reaction contained a total volume of 50 μl with 1 U of Taq polymerase mixture (PCR Supermix High Fidelity, Gibco BRL Inc., Rockville, MD) and 0.2 μM primers with the following parameters: 2 min initial denaturation at 94°C; 30 s denaturation at 94°C, 30 s annealing at 58°C, 2 min extension at 72°C (30 cycles) and 5 min final extension at 72°C. Primers were designed on the basis of the reported chicken myostatin mRNA sequence (GeneBank Accession number, AF019621). The forward primers for unprocessed form of and C-terminal myostatin cDNA were 5'-ATGCAAAAGCTAGCAGTCTATG-3' and 5'-GAGGTCAGAGTTACAGACACA-3', respectively, and the reverse primer for both forms was 5'-TCATGAGCACCAGGCAACGATC-3'. These primer combinations were designed to yield a PCR product corresponding to bases 1-1125 and 760-1125 of chicken myostatin mRNA sequence. Appendix 2 and 3 show that the agarose gel electrophoresis of the PCR product.
2.2.2 Construction of myostatin fragment expression vector

The amplified myostatin cDNA fragments were purified from the PCR reaction mixture using a commercially available PCR purification kit (Amersham Pharmacia, Piscataway, NJ), and subsequently inserted into a cloning vector (pCR®T7/NT-TOPO, Invitrogen, Carlsbad, CA) following the manufacture's instruction. Appendix 4 shows the map of Topo cloning site. The cDNAs were introduced by heat shock into competent E. coli (TOP10F’ One Shot®, Invitrogen,) for the characterization of construct, propagation and maintenance. Clones were identified for correct insertion using PCR and restriction analysis of the plasmids. Appendix 5 and 6 show the selection of correctly inserted clones. After the initial screening, plasmids of selective clones were subject to DNA sequence analysis for final confirmation of correct orientation.

2.2.3 Expression of recombinant myostatins

Plasmids containing the myostatin DNA fragments were isolated, and were introduced by heat shock into expression competent E. coli [BL21 (DE3) pLysS One Shot®, Invitrogen, Carlsbad, CA] haboring the lambda DE3 lysogen that carries the T7 RNA polymerase under the control of the lacUV5 promoter. The transformation mixture (250 µl) was added to LB medium (10 ml) containing 100 µg/ml ampicillin and 34 µg/mL chloramphenicol for overnight grow at 37°C with vigorous shaking. This culture was used to inoculate prewarmed 200 ml LB medium containing 100 µg/mL ampicillin and 34 µg/mL chloramphenicol. Myostatin expression was induced at an OD$_{600}$ of 0.6-0.7 by adding IPTG to 1 mM. Expression of myostatin was monitored during incubation by SDS-PAGE analysis. Typically, 4 hour incubation was enough to
induce maximum expression. After 4 hours, cells were harvested by centrifugation at 5,000 g for 20 min and stored frozen for later use.

The expression of the recombinant myostatin fragments (expected MW, 46 kD for prepropeptide of myostatin and 18 kD for mature form of myostatin) was confirmed by SDS-PAGE analysis. Figure 2.1 show predicted amino acid sequence of recombinant chicken myostatin fragments.

2.2.4 Isolation of inclusion bodies

The pellets from 200 mL of culture were resuspended in 40 mL of 20 mM phosphate buffer containing 500 mM NaCl (pH 7.8), and incubated at room temperature for 15 min after adding 100 μg/mL egg white lysozyme. Cells were lysed by combination of sonication and 3 cycles of flash freeze in liquid nitrogen and thaw at 37°C. The insoluble material was pelleted by centrifugation at 5,000 g for 20 min, followed by twice washing in a buffer (lysis buffer) containing 50 mM potassium phosphate, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100, 10 mM imidazole (pH 7.8). The pellet was washed again twice with distilled water to remove salts and detergents. About 12 mg of purified inclusion body proteins were harvested from 200 mL culture. The final sediment was used for solubilization and refolding.

2.2.5 Purification of recombinant protein

Myostatin inclusion bodies containing prepropeptide of myostatin and mature form of myostatin were solubilized (3 mg protein/mL) in commercially available 50 mM CAPS buffer (pH 11) containing 0.3% N-lauroylsarcosine and 1 mM DTT (Protein Refolding Kit, Novagen, WI). Inclusion body proteins were fractionated by SDS-PAGE. Myostatin bands were cut out and electro-eluted in order to prepare purified myostatins.
The electro-eluted myostatin proteins were dialyzed first in 20 mM Tris buffer (pH 8.5) containing 1 mM DTT and second in the same buffer without DTT at room temperature. Dialysis volume was 50 times the volume of sample, and each dialysis was done at 4°C with dialysis buffer change at 5 hr intervals.

2.2.6 Immunization of rabbit and serum collection

New Zealand White female rabbits weighing approximately 2 kg were subcutaneously injected with antigen suspended in Complete Freund’s adjuvant solution (1 mg/ml). The Complete Freund’s adjuvant and antigen were mixed in 1:1 ratio. Three additional boosters were subcutaneously given 2-3 weeks apart after primary immunization. Final bleedings was done 4 days after the last booster. Collected blood was placed at 4°C overnight and centrifuged at 10,000g for 10 minute to collect sera.

2.2.7 Enzyme-Linked Immunosorbent Assay (ELISA)

Coating antigen was the recombinant chicken myostatin prepro-myostatin and mature form myostatin that were purified by electro elution of SDS-PAGE fractionated band. 50 µl of recombinant myostatin proteins (50 µg/ml) were added to each well of micro-ELISA plate, and the plate was covered with lid and incubated for 2 hours at room temperature. The plate was washed with phosphate buffered saline (PBS: 20mM sodium phosphate, 15mM NaCl, pH 7.4), and 100 µl of 1% BSA in PBS was added to each well, then incubated at room temperature for 1 hour. The plate was washed with PBS, then 50 µl of various diluted antiserum was added to each well containing antigen for 1 hour at room temperature. After incubation, the plate was washed 3 times with PBS-0.05 % Tween-20, then 50 µl of anti-rabbit IgG alkaline phosphatase conjugate in PBS-0.05 % Tween-20 (1:10,000) were added, and incubated at room temperature. The
plate was washed 3 times with PBS-0.05 % Tween-20, and 50 μl of 4- nitrophenyl phosphate (pNPP) was added and incubated for 30 minutes in the dark at room temperature. The reaction was stopped by adding 25 μl of 3N NaOH, and O.D. was measured at 405 nm on a microplate reader.

### 2.2.8 Affinity purification of anti-myostatin IgG

Anti-myostatin antibody was purified by Protein A affinity chromatography using a commercially available system (Affi-gel protein A MAPS® II kit, Bio-Rad). The sera containing anti-myostatin antibodies were diluted with buffer (MAPS II binding buffer, BioRad) and filtered through a 0.22 μ filter. The diluted solution was loaded into a Protein A column equilibrated with 5 bed volumes of the binding buffer, then column was washed with 15 volumes of the binding buffer. Antibody was eluted with 5 bed volumes of the elution buffer (MAPS II elution buffer, BioRad), and 1 ml fraction was collected into tubes containing 1.6 ml of 1M Tris buffer (pH 8.0) during elution. The purified IgGs were analyzed by SDS-PAGE under reducing condition.

### 2.2.9 Protein assay

Sample protein concentrations was determined by either the Lowry method (1951) or the Bradford method (1976) using bovine serum albumin as a standard.

### 2.2.10 Sodium dodecyl sulphate and polyacrylamide gel electrophoresis (SDS-PAGE) analysis

SDS-PAGE was performed on mini gels (9 x10 cm) by the method of Laemmli (1970) using 18% polyacrylamide gels in the presence of 0.1% SDS under reducing or non-reducing conditions. The gels were either stained with Coomassie blue or subject to electophoretic transfer onto a nitrocellulose membrane for Western blot analysis.
2.2.11 Western blot analysis

Antibody binding specificity was examined using Western transfer and immunoblotting. Tissue samples from chicken embryo and adult chicken were homogenized in 10 volumes of sodium phosphate buffer (10 mM sodium phosphate monobasic and 10 mM sodium phosphate dibasic without SDS, pH 7.0). Total muscle homogenate were subjected to 12.5% SDS-PAGE gel. Proteins were electrophoretically transferred onto nitrocellulose membrane while immersed in Towbin buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.1% SDS). After transfer, membranes were blocked with TBS (125 mM NaCl, 25 mM Tris, pH 8.0) buffer containing 0.5% Tween 20 for 1 hour at room temperature. Membranes were incubated for 1 hour at room temperature in TBS buffer containing polyclonal anti-myostatin antibodies (0.84 μg/ml). After washing, membrane was reacted to alkaline phosphatase conjugated anti-rabbit IgG (1:5,000 times dilution; Sigma, St. Louis, MO) for 1 hour at room temperature. After washing, blots were developed using nitroblue tetrazolium and bromo-chloro-indolyl phosphate (BCIP/NBT).
2.3 RESULT

2.3.1 Expression of recombinant chicken myostatins

Figure 2.2 and 2.3 show the expression of recombinant chicken mature form of myostatin (C-terminal sequence) and prepro-myostatin (unprocessed sequence), respectively, at various times after induction with IPTG. The expression of 18 kD C-terminal recombinant myostatin (Figure 2.2) and 46 kD prepro-myostatin (Figure 2.3) were clearly visible at 1 hour after induction, and no further increase in protein expression was observed with prolonged incubation. During the induction of prepro-myostatin, the expression of recombinant myostatin protein appeared to suppress the growth of *E. coli* as demonstrated by the weaker staining intensity of the IPTG added culture than that of the control culture.

2.3.2 Purification of recombinant chicken myostatins

Myostatin proteins were purified by electro-elution of myostatin containing bands obtained from SDS-PAGE fractionation. Figure 2.4 shows the result of SDS-PAGE analysis of the purified chicken myostatin proteins. Other than the purified prepro-myostatin and mature form of myostatin, no other bands were visible in the SDS-PAGE analysis. These purified proteins were used as immunogens in polyclonal antibody production and as coating antigens in ELISA.

2.3.3 Production of antibodies against chicken myostatins

Table 2.1 summarizes the titer values after immunization. When the titer values were compared between the 1st immunization and the 4th immunization, the titer value of the 4th immunization was higher than that of the 1st immunization for both against the prepro-myostatin and mature form of myostatin, indicating that the amount of
antibody production was highest at 4th immunization.

2.3.4 Affinity purification of anti-myostatin IgG and titer determination of the purified anti-myostatin antibodies

Figure 2.5 shows SDS-PAGE analysis of purified IgG. Albumin (molecular weight at 66kD) present in sera was removed after Protein A affinity chromatography. The 50 kD heavy chain (arrow 1) and 25 kD light chain (arrow 2) of IgG have become dominant bands in SDS-PAGE after the purification of the sera. Figure 2.6 shows the titer determination of purified anti-myostatin antibodies. When the affinity of both antibodies was tested in ELISA using the mature form of myostatin as a coating antigen, the polyclonal antibody against the mature form of myostatin has a higher affinity than polyclonal antibody against prepro-myostatin to the coating antigen (Figure 2.6 A). When the affinity of both antibodies was tested in ELISA using the prepro-myostatin as a coating antigen, no significant difference in affinity to the coating antigen was observed (Figure 2.6 B). The results imply that the polyclonal antibody against prepro-myostatin had population of antibodies that probably have affinity to the prodomain of prepro-myostatin, in addition to the population of antibodies that have affinity to the mature form of prepro-myostatin.

2.3.5 Western blot analysis of binding characteristics of polyclonal anti-myostatin antibodies

Figure 2.7 shows the results of Western blot analysis of the binding affinity of polyclonal antibodies against prepro-myostatin and mature myostatin. Both polyclonal antibodies had strong affinity to both the prepro-myostatin and the mature myostatin. While various proteins in liver showed affinity to both the anti-prepro-myostatin
antibody and the anti-mature myostatin antibody, proteins at 50, 30, 25 and 20 kD demonstrated a stronger affinity to the antibodies than the other proteins. No significant difference in binding pattern was recognizable between the two polyclonal antibodies in the liver tissue. When skeletal muscle proteins were probes with the anti-mature myostatin antibody, proteins at 50, 30 and 25 kD showed affinity (Figure 2.7 C). Based on the migration pattern in the blot, these proteins appeared to be the same proteins as those observed in liver. When skeletal muscle proteins were probed with the anti-prepro-myostatin antibody, the proteins at 50, 30 and 25 kD that demonstrated affinity to the anti-mature myostatin antibody also demonstrated affinity to the anti-prepro-myostatin antibody (Figure 2.7 B). In addition, a protein at 37 kD appeared to have specific affinity to the anti-prepro-myostatin antibody but not to the anti-mature myostatin antibody (Figure 2.7 B). Since in liver the 37 kD band was not recognized by either of the antibodies, the 37 kD protein appeared to be a unique protein present in skeletal muscle but not in liver.

To further examine whether the 37 kD protein is uniquely present in skeletal muscle but not any other tissue, various tissue samples were probed with anti-prepro-myostatin in Western blot analysis (Figure 2.8). Samples from adult and 21 day embryonic chicken were used to examine whether there exists any age-related difference in the intensity of 37 kD protein. In addition to leg and breast muscles, the 37 kD protein also appeared to be present in heart muscle, but not in liver, crop, kidney, spleen, brain tissues from both adult and 21 day embryonic chicken. The intensity of 37 kD protein was greater in skeletal muscles than the heart muscle, and adult muscle showed a greater intensity of the 37 kD protein than the 21 day embryonic muscle. The presence
of 37 kD protein in skeletal muscles from other species was examined using Western blot analysis (Figure 2.9). The 37 kD protein was also present in skeletal muscles of mice, pigs and cattle in addition to chicken skeletal muscle. Western blot analysis was also performed with commercially available antibodies (Santa Cruz Biotechnology Inc. CA) generated against C-terminal (C20) and N-terminal (N19) myostatins (Figure 2.10). Both antibodies showed affinity to our recombinant prepro-myostatin and mature myostatin. Considering that the anti-N19 myostatin antibody was generated against a peptide fragment present in prodomain region of myostatin, the binding of this antibody to our recombinant mature myostatin was unexpected. Repeat of western blot analysis with the anti-N19 myostatin antibody resulted in the same binding to the mature myostatin, indicating that the binding was not an experimental error. The extreme similarity in the binding pattern of these antibodies (Figure 2.10) leads us to suspect whether the two antibodies were raised against the same antigen. Since we did not make any efforts to resolve this question, it probably is desirable not to discuss the result with the anti-N19 antibody. In skeletal muscle and liver, proteins at 17, 25 and 30 kD showed strong affinity to the commercial anti-myostatin antibody. Close examination of the western blot results probed with antibodies generated in our study indicates that the three proteins recognized by the commercial anti-myostatin antibody were also recognized by our anti-myostatin antibodies with different affinity. The binding pattern of the commercial antibody in skeletal muscle did not differ from that in liver.
2.4 DISCUSSION

Myostatin has been indicated to be produced as a precursor protein composed of a signal sequence, an N-terminal prodomain and a C-terminal mature domain (Zimmers et al., 2002). The mature form of myostatin is formed upon removal of the N-terminal prodomain by proteolysis at the tetrabasic (RSRR) site (Lee and McPherron, 2001). The presence of a 52 kD unprocessed and a 15 kD active form of myostatin was demonstrated in Chinese hamster ovary (CHO) cells expressing myostatin (McPherron et al., 1997). Furthermore, when myostatin was purified from a conditioned medium of myostatin expressing CHO cells, a 36 kD latent peptide was identified (Lee and McPherron, 2001). The mature form of myostatin as well as the precursor form of myostatin appears to form a disulfide-linked dimmer like many other members of the TGF-β superfamily (McPherron et al., 1997; Lee and McPherron, 2001), but myostatin prodomain did not form disulfide-linked dimmer (Lee and McPherron, 2001; Thies et al., 2001). In mouse serum, two myostatin-immunoreactive proteins (~12 kD and ~36 kD) were recently separated using immuno-precipitation method, and using LC-MS-MS method the 12 kD and 36 kD proteins were identified as mature myostatin and myostatin prodomain, respectively (Hill et al., 2002; Zimmers et al., 2002). Since myostatin mRNA is almost exclusively expressed in skeletal muscles, the mature myostatin and myostatin prodomain present in serum are likely from secretions from skeletal muscles. Even though skeletal muscle is presumed to be the major site for myostatin production, the demonstration of the presence of myostatin protein in skeletal muscle has been equivocal, and purification of myostatin from skeletal muscle has not been achieved yet.

In the present study, we generated anti-myostatin antibodies by immunizing rabbit
against purified recombinant prepro-myostatin and mature form of myostatin. These antibodies demonstrated strong binding affinity to the recombinant myostatins in both ELISA and western blot analysis. In western blot analysis with skeletal muscles, the polyclonal anti-mature myostatin showed affinity to proteins at around 50, 30 and 20 kD. Similar to our results, Gonzalez-Cadavid et al. (1998) reported that their anti-myostatin antibody raised against a peptide fragment from mature myostatin had strong affinity to a 30 kD protein in rat and mouse skeletal muscles. Other studies also reported the presence of 50 kD and 20 kD of myostatin immuno-reactive proteins in skeletal muscles in western blot analysis (Sharma et al., 1999; Mendler et al., 2000). Considering that myostatin can be present in many different forms including 52 kD non-processed form, 36 kD prodomain and 12.5 kD mature form, it is tempting to assume that some of the above proteins that had affinity to our anti-mature myostatin antibody probably belong to one of the different forms of myostatin. However, our current results do not support the assumption. The above proteins were also detected in liver and other tissues (data not shown), conflicting with evidence that skeletal muscle is the major site of myostatin production. In addition, when western blot was performed with blots from non-reducing SDS-PAGE, the molecular weights of the above proteins stayed the same (data not shown), conflicting with the disulfide bridge forming property of unprocessed and mature myostatins. According to a recent study by Zimmers et al. (2002), anti-myostatin antibody has strong affinity to various proteins in a western blot condition that were able to detect mature myostatin in mouse serum. In fact, the nonspecific bindings were dominant bindings occurred in their western blot analysis, demonstrating the difficulty of detecting myostatin in western blot analysis without incurring non-specific
bindings. Taken together, it is quite likely that the previously reported myostatin immuno-reactive proteins present in skeletal muscles (Gonzalez-Cadavid et al., 1998; Sharma et al., 1999; Mendler et al., 2000) as well as the 50, 30 and 20 kD proteins in our study are non-myostatin proteins that had affinity to anti-myostatin antibodies. It appears, therefore, that measuring myostatin in skeletal muscles using western blot analysis with our anti-mature myostatin antibody probably requires additional steps that concentrate myostatin and eliminate major non-specific binding proteins from skeletal muscles. Furthermore, caution needs to be exercised in interpreting results in which the myostatin concentration was estimated by western blot analysis without rigorous validation of the binding characteristics of the anti-myostatin antibody used in the analysis.

Similar to the anti-mature myostatin antibody, the anti-prepro-myostatin demonstrated affinity to proteins at 50, 30 and 25 kD both in liver and skeletal muscles. However, in addition to those proteins, this antibody had a strong affinity to a 37 kD protein in skeletal muscles but not in liver. Further western blot analysis with various tissues revealed that the 37 kD protein uniquely present in skeletal and cardiac muscles but not in other tissues including liver, crop, kidney, brain and spleen. Considering that the polyclonal anti-prepro-myostatin antibody was raised against the recombinant chicken prepro-myostatin myostatin containing the prodomain and mature form, and the molecular weight of the latent peptide of myostatin is known to be 36 kD (Lee and McPherron et al., 2001; Wehling et al., 2000; Zhu et al., 2000; Hill et al., 2002), the 37 kD protein is postulated to be a prodomain of chicken myostatin. In support of the current postulation, the commercial anti-myostatin antibody raised against a peptide
fragment from mature myostatin did not show affinity to the 37 kD protein. In addition to skeletal muscles, the 37 kD protein was present in cardiac muscles with much lower concentration as compared to skeletal muscles, indicating a potential production of myostatin in cardiac tissue. In support of the presence of myostatin in cardiac muscle, Sharma et al. (1999) demonstrated the expression of myostatin mRNA in cardiac muscle.

When the presence of 37 kD protein in skeletal muscles from other species was examined, the 37 kD protein was also present in skeletal muscles of mice, pigs and cattle. It was initially rationalized that the anti-chicken prepro-myostatin antibody would not show affinity to the 37 kD protein in skeletal muscles of other species if the 37 kD is indeed the myostatin prodomain considering the sequence divergency existing in the prodomain of myostatin among different species. On the other hand, however, the anti-chicken prepro-myostatin antibody would show affinity to the 37 kD protein in skeletal muscles of other species if the antibody recognize epitopes shared by the myostatin prodomain from different species. Since many homologous sequence regions are present in myostatin prodomain even though it is much more divergent among species as compared to mature myostatin, it appears possible that many populations of anti-chicken prepro-myostatin antibody would raised against epitopes that are homologous among species.

Since the results suggested that the anti-prepro-myostatin antibody has affinity to the 37 kD myostatin prodomain, we examined the effect of in ovo administration of the antibody on posthatch chicken growth in a small pilot experiment (Appendix 13). The hypothesis was that the anti-prepro-myostatin antibody binds to myostatin prodomain, thus suppress the prodomain’s inhibitory effect of the biological activity of
myostatin, resulting in stimulation of myostatin's biological activity. If the above hypothesis is true, we expected that the chicks injected with antibodies in ovo would show diminished growth because of the suppressed muscle development during embryonic stage. Interestingly, the mean body weight of chicks injected with antibody was lower as we hypothesized. However, the number of chick used in those trials was too small to reach to any meaningful conclusion. Further studies are probably required to confirm the above preliminary results.

In conclusion, we generated anti-prepro-myostatin and anti-mature myostatin antibody by immunizing rabbit with recombinant chicken prepro-myostatin and mature myostatin. The anti-mature myostatin antibody could not recognize myostatin present in skeletal muscle. In contrast, the anti-prepro-myostatin antibody showed a specific binding to a 37 kD protein in skeletal muscle, which appears to be a myostatin prodomain.
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gene MRF4 results in up-regulation of myogenin and rib anomalies. Genes and


Table 2.1. Average value of titer by ELISA after immunization

<table>
<thead>
<tr>
<th>Immunization</th>
<th>OD$_{405}$</th>
<th>Prepro-myostatin</th>
<th>Mature form of myostatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1^{st}$</td>
<td>0.303</td>
<td>0.271</td>
<td></td>
</tr>
<tr>
<td>$2^{nd}$</td>
<td>0.353</td>
<td>0.275</td>
<td></td>
</tr>
<tr>
<td>$3^{rd}$</td>
<td>0.328</td>
<td>0.235</td>
<td></td>
</tr>
<tr>
<td>$4^{th}$</td>
<td>0.452</td>
<td>0.303</td>
<td></td>
</tr>
</tbody>
</table>

Coating antigen was prepro-myostatin purified by electro-elution of SDS-PAGE fractionated band. Sera were diluted by 1,000 times. The ODs represent background substrated values.
Figure 2.1. Predicted amino acid sequences of recombinant chicken myostatin fragments

Amino acid sequence originated from cloning vector is red letters. C-terminal fragment is blue letters. The expected proteolytic processing site is shown in bold characters and the conserved cysteine residues in the C-terminal region are underlined in small characters.
Figure 2.2. SDS-PAGE analysis of myostatin expression (366 bp)

500 μl of culture were collected every hour for 6 hours during fermentation, then cell pellets prepared by centrifugation were mixed with 80 μl SDS-PAGE loading buffer, and 5 μl of each sample was applied on an 18% SDS-polyacrylamide gel after boiling for 5 minutes. Arrow indicates the expression of recombinant myostatin. Lane 1, protein standard; lanes 2, 4, 6, 8, 10, 12 and 14 - 0 mM IPTG; lanes 3, 5, 7, 9, 11, 13 and 15 - 1 mM IPTG
Figure 2.3. SDS-PAGE analysis of myostatin expression (1125 bp)

500 μl of culture were collected at various time as indicated above during fermentation, then cell pellets prepared by centrifugation were mixed with 80 μl SDS-PAGE loading buffer, and 5 μl of each sample was applied on an 18% SDS-polyacrylamide gel after boiling for 5 minutes. Arrow indicates the expression of recombinant myostatin. Lane 1, protein standard; lanes 4, 6, 8, 10 and 12 - 0 mM IPTG; lanes 3, 5, 7, 9 and 11 - 1 mM IPTG
Figure 2.4. SDS-PAGE analysis of purified recombinant myostatin used for immunogens

Myostain inclusion bodies containing prepro and mature forms were solubilized in a commercially available solution (Protein Refolding Kit, Novagen, WI), then proteins were fractionated by SDS-PAGE. Myostatin bands were cut out and electro-eluted in order to prepare purified myostatins. The electro-eluted myostatin proteins were dialyzed first in 20 mM Tris buffer (pH 8.5) containing 1 mM DTT and second in the same buffer without DTT.
Figure 2.5. SDS-PAGE analysis of affinity purified anti-myostatin IgG

Anti-myostatin antibodies were purified by Protein A affinity chromatography using a commercially available system (Affi-gel protein A MAPS® II kit, Bio-Rad). The sera containing anti-myostatin antibodies were diluted with dilution buffer and filtered through a 0.22 μ filter. The diluted solution was loaded into a Protein A column equilibrated with 5 bed volumes of the binding buffer, then column was washed with 15 volumes of the binding buffer. Antibody was eluted with 5 bed volumes of the elution buffer while collecting 1 ml fraction into 1.6 ml of 1M Tris buffer (pH 8.0). The purified IgGs were analyzed by SDS-PAGE under reducing condition.

Lane 1, protein standard; Lane 2, 100 times diluted serum from immunization with prepro-myostatin; Lane 3, 100 times diluted serum immunization with mature form; Lane 4, 50 times concentrated purified IgG against prepro-myostatin; Lane, 50 times concentrated purified IgG against mature myostatin.
Coating antigen:
Mature form of myostatin

- Prepro-myostatin antibody
- Mature form of myostatin antibody
- Control

Coating antigen:
Prepro-myostatin

- Prepro-myostatin antibody
- Mature form of myostatin antibody
- Control
Figure 2.6. Titer determination of protein A purified IgGs by ELISA: A) mature form myostatin as a coating antigen B) prepro-myostatin as a coating antigen

Coating antigens were the recombinant chicken prepro-myostatin and mature myostatin that were purified by electro elution of SDS-PAGE fractionated bands. Protein concentration in the coating antigen solution was 50 µg/ml (2.5 µg/well). Primary antibodies were anti-prepro-myostatin and anti-mature myostatin antibodies. Anti-rabbit IgG alkaline phosphate conjugate was used as a secondary antibody (1:10,000), and 4-nitrophenyl phosphate (pNPP) was used as a substrate.
Figure 2.7. Western blot analysis of binding characteristics of polyclonal anti-myostatin antibodies to recombinant chicken myostatins, liver and skeletal muscle proteins

SDS-PAGE purified recombinant chicken myostatins and proteins from chicken leg muscle and liver were fractionated under reduced condition on a 12.5% SDS-PAGE (A), then blotted onto nitrocellulose membrane and probed with polyclonal antibodies against prepro-myostatin (B) and polyclonal antibody against mature myostatin (C). Chicken tissue samples from 19 day old embryo were homogenized in a phosphate buffer (pH 7.4). The homogenates were centrifuged at 25,000 g for 30 minute at 4°C to separate the soluble and insoluble fractions. The protein concentration in the SDS-PAGE loading sample was adjusted to before loading 1 μg/μl, and 14 μl was loaded in each well. The primary antibody concentration was 0.84 μg/ml in this Western blot analysis. The secondary antibody was diluted by 1:5,000 times with PBS buffer.

LH= Liver Homogenate, LS=Liver Supernatant, LmH=Leg muscle Homogenate, LmS=Leg muscle Supernatant
Figure 2.8. Western blot analysis of binding characteristics of prepro-myostatin antibody in various tissues of chicken

Tissue samples (liver, heart, breast muscle, leg muscle, crop, kidney, spleen and brain) from 21 day old embryo and 3 weeks old chicken were homogenized in 10 mM sodium phosphate buffer (pH 7.0). Then, the homogenates were centrifuged at 25,000 g for 30 minute at 4°C, and supernatant were collected. The protein concentration of supernatant was adjusted to 2.5 μg/μl before loading into SDS-PAGE gel, and 15 μl of the supernatant was loaded into each well. The primary antibody concentration was 0.84 μg/ml in Western blot analysis. The secondary antibody was diluted by 1:5,000 times with PBS buffer.
Figure 2.9. Western blot analysis of binding characteristics of anti-prepro-myostatin antibody in skeletal muscle from chicken, mice, pig and cattle

Muscle samples were homogenized in 10 mM sodium phosphate buffer (pH 7.0). Then, the homogenates were centrifuged at 25,000 g for 30 minute at 4°C, and the supernatants were collected sample. The protein concentration of supernatant was adjusted to 2.5 μg/μl before loading into SDA-PAGE gel, and 15μl of the supernatant was loaded into each well. The primary antibody concentration was 0.84μg/ml in Western blot analysis. The secondary antibody was diluted by 1:5,000 times with PBS buffer.
A) < SDS-PAGE >

B) < Western blot : GDF-8 (C-20) >

C) < Western blot : GDF-8 (N-19) >
2.10. Western blot analysis of binding characteristics of commercially available polyclonal antibodies generated against C-terminal (GDF-8, C20) and N-terminal (GDF-8, N19) in chicken liver and skeletal muscle homogenates

SDS-PAGE purified recombinant chicken myostatins and proteins from chicken muscle and liver were fractionated under reduced condition on a 12.5% SDS-PAGE (A), then blotted onto nitrocellulose membrane and probed with commercially available (Santa Cruz Biotechnology Inc. CA) myostatin antibodies generated against C-terminal (GDF8-C20) (B) and N-terminal (GDF8-N19) myostatin (C). Chicken tissue samples from 19 day old embryo were homogenized in a phosphate buffer (pH 7.4). The protein concentration in the SDS-PAGE loading sample was adjusted to 1 μg/μl before loading, and 14 μl was loaded in each well. The primary antibody was diluted by 1:500 times, and the secondary antibody was diluted by 1:5,000 times with PBS buffer.

LH= Liver Homogenate, BmH=Breast muscle Homogenate, LmH=Leg muscle Homogenate
APPENDIX 1

Fractionation of total RNA in 1% Agarose gel electrophoresis

Lanes 1–4 are total RNA separated by Trizol solution, and lane 5–7 is total RNA separated with USB kit. Lanes 1 and 5 are from chicken heart muscle, lanes 2, 3, and 6 are from chicken skeletal muscle, lane 4 and 7 are from chicken liver.
APPENDIX 2

Agarose gel (1.2%) electrophoresis of PCR products (1125 bp) obtained from RT-PCR using RNAs isolated from both chicken heart and skeletal muscles

DNA markers are 2000, 1500, 1000, 700, 500, 400, 300, 200 and 100 bp (lane 1). Lane 2, chicken skeletal muscle total RNA; lane 3, chicken skeletal muscle mRNA; lane 4, chicken heart total RNA; lane 5, chicken heart mRNA; lane 6, chicken liver total RNA.
APPENDIX 3

Agarose gel (1.2%) electrophoresis of PCR product (366 bp) obtained from RT-PCR using mRNAs isolated from chicken skeletal muscle
APPENDIX 4

Map of Topo cloning site

**T7 promoter**

1  GATCTCGATC CCGGAATTT AATACGACT C ACTATAGGGA GACCACAACG

\[Nde\ I\]

51  GATCTCGATC CCGGAATTT AATACGACT C ACTATAGGGA GACCACAACG

\[Nhe\ I\]

100  ATG CGG GGT TCT CAT CAT CAT CAT CAT GGT ATG GCT AGC ATG

**6X His Region**

144  ACT GGT GGA CAG CAA ATG GGT CGG GAT CTG TAC GAC GAT GAC GAT

**Xpress epitope**

190  AAA GAT CCA ACC CTT PCR Product AGGGCGA GAATTCGAAG CTT

\[BamHI\]

\[EcoRI\]

\[BstBI\]

\[HindIII\]

\[EK cleavage site\]
APPENDIX 5

PCR identification of positive transformants with correct orientation of inserted fragment (366 bp)

Purified plasmids (about 200 ng) from selected clones were subject to PCR in a total volume of 50 µl with 1 U of Taq polymerase mixture (PCR Supermix High Fidelity, Gibco BRL Inc., Rockville, MD) and 0.2 µM primers. Two kinds of primer combination were used. One combination (lane 3, 5 and 7) was designed to amplify the insert (366 bp): forward primer (5'-GAGGTCAGAGTTACAGACACA-3') and reverse primer (5'-TCATGAGCACCCGCAACGATC-3'). In the other primer combination (lane 2, 4 and 6), the forward primer was from T7 promoter priming site (TAATACGACTCACTATAGGG) and reverse primer was the same as above, designed to yield 554 bp with correct orientation of inserted fragment. Five µls of PCR products were loaded in a 2% agarose gel for size separation. Lane 1 represents size ladder (2000, 1500, 1000, 700, 500, 400, 300, 200, 100, and 50 bps); lanes 2 and 3, 4 and 5, 6 and 7 represent clones 1, 2, and 3, respectively. Clones 2 and 3 were identified as having a correct orientation.
APPENDIX 6

PCR identification of positive transformants with correct orientation of inserted fragment (1125 bp)

Purified plasmids (about 200 ng) from selected clones were subject to PCR in a total volume of 50 μl with 1 U of Taq polymerase mixture (PCR Supermix High Fidelity, Gibco BRL Inc., Rockville, MD) and 0.2 μM primers. Two kinds of primer combination were used. One combination (lane 3, 5 and 7) was designed to amplify the insert (1125 bp): forward primer (5′-ATGCAAAAGCTAGCAGTCTATG-3′) and reverse primer (5′-TCATGAGCACCCGCAACGATC-3′). In the other primer combination (lane 2, 4 and 6), the forward primer was from T7 promoter priming site (TAATACGACTCACTATAGGG) and reverse primer was the same as above, designed to yield 1311 bp with correct orientation of inserted fragment. Five μls of PCR products were loaded in a 2% agarose gel for size separation. Lane 1 represent size ladder (2000, 1500, 1000, 700, 500, 400, 300, 200, 100, and 50 bps); lanes 2 and 3, 4 and 5, 6 and 7 represent clones 2, 6 and 10, respectively. All the clones were identified as having a correct orientation.
APPENDIX 7

Titer determination by ELISA, A) anti-mature myostatin B) anti-prepro- myostatin

A)

Serum against anti-mature myostatin

B)

Serum against anti-prepropeptide myostatin
Coating antigen was the recombinant chicken prepro-myostatin that was purified by electro elution of SDS-PAGE fractionated band. Protein concentration in the coating antigen solution was 46 µg/ml (2.3 µg/well). Anti-rabbit IgG alkaline phosphatase conjugate was used as a secondary antibody (1:10,000), and 4-nitrophenyl phosphate (pNPP) was used as a substrate.
APPENDIX 8

Solubility of 37 kD protein at different pH

A) <SDS-PAGE of whole homogenate>

B) <Western blot of whole homogenate>

C) <SDS-PAGE of supernatant>

D) <Western blot of supernatant>
To examine the solubility of the 37 kD protein various buffer pH, breast muscle sample from 21 day old embryo were homogenized in phosphate buffer solutions with pH at 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. Supernatants were collected after centrifugation (1,000g), and proteins were fractionated by SDS-PAGE, transferred onto nitrocellulose membrane. The presence of 37 kD protein was examined by immunoblotting probed with anti-prepro-myostatin antibody. The results demonstrated that the 37 kD protein in soluble at pH beyond 6.0, but not at below 5.0.
APPENDIX 9

Effect of centrifugation force on the extraction of 37 kD protein

A)  

< SDS-PAGE of supernatant>

B)  

< Western blot with anti-prepro-myostatin antibody >

C)  

< Western blot with anti-mature myostatin antibody >
To examine the effect of centrifugation on the extraction of 37 kD protein, breast muscle sample from 21 day old chicken embryo was homogenized in buffer solution with pH indicated in the figure, then supernatants were collected after centrifugation at speeds as indicated in the figure. The presence of 37 kD protein in the supernatant was examined using Western blot analysis probed with the anti-prepro-myostatin antibody (B) and the anti-mature myostatin antibody (C). The results demonstrate that the centrifuge force did not affect the extraction of the 37 kD protein.
APPENDIX 10

Examination of the presence of the 37 kD protein in the whole homogenate and supernatant of various tissues from 21 day old chicken embryo

A) < SDS-PAGE of whole homogenate >

B) < Western blot of whole homogenate >

C) < SDS-PAGE of supernatant >

D) < Western blot of supernatant >
APPENDIX 11

Examination of the binding sensitivity of the polyclonal anti-myostatin antibodies at various dilutions in liver and skeletal muscle

A) <SDS-PAGE>

B) <Western blot with anti-prepro-myostatin antibody>

C) <Western blot with anti-mature myostatin antibody>
Chicken skeletal muscle samples from 19 day old embryo were homogenized in 10 mM sodium phosphate buffer (pH 7.4). Samples were fractionated under reduced condition on a 12.5% SDS-PAGE (A), then blotted into nitrocellulose membrane and probed with polyclonal antibody against prepro-myostatin (B) and polyclonal antibody against mature form (C). The protein concentration in the SDS-PAGE loading sample was 1 μg/μl, and 10 μl was loaded in each wall.

LH=Liver Homogenate, LmH=Leg muscle Homogenate
APPENDIX 12

Examination of the binding of anti-myostatin antibodies in various amount of skeletal muscle

A)  

< SDS-PAGE >

B)  

< Western blot with anti-prepro-myostatin antibody >

C)  

< Western blot with anti-mature myostatin antibody >
Chicken leg muscle and liver samples from 19 day old embryo were homogenized in 10 mM sodium phosphate buffer (pH 7.4). Samples were fractionated under reduced condition on a 12.5% SDS-PAGE (A), then blotted into nitrocellulose membrane and probed with polyclonal antibody (1:1,000) against prepro-myostatin (B) and polyclonal antibody (1:1,000) against mature form (C). The protein concentration in the SDS-PAGE loading sample was 1 \mu g/\mu l.

LmH=Leg muscle Homogenate, BmH= Breast muscle Homogenate
APPENDIX 13

Posthatch growth after in ovo injection of anti-prepro-myostatin antibody

Table 1. Number of eggs used and hatched in trial I (14 eggs)

<table>
<thead>
<tr>
<th></th>
<th>No injection: 7 eggs</th>
<th>Injection (antibody): 200μl/eggs -7eggs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hatching</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Failed hatching</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Survival % posthatch</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 2. Body weight at 22 day posthatch in trial I

<table>
<thead>
<tr>
<th>Control</th>
<th>total body weight (g)</th>
<th>breast muscle (g)</th>
<th>% of breast muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>630</td>
<td>92.9</td>
<td>14.75</td>
</tr>
<tr>
<td>2</td>
<td>650</td>
<td>97.9</td>
<td>15.06</td>
</tr>
<tr>
<td>3</td>
<td>658</td>
<td>92.4</td>
<td>14.04</td>
</tr>
<tr>
<td>4</td>
<td>750</td>
<td>120.8</td>
<td>16.11</td>
</tr>
<tr>
<td>5</td>
<td>643</td>
<td>87.3</td>
<td>13.58</td>
</tr>
<tr>
<td>Avg.</td>
<td>666.2</td>
<td>98.26</td>
<td>14.71</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>total body weight (g)</th>
<th>breast muscle (g)</th>
<th>% of breast muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>604</td>
<td>81.5</td>
<td>13.49</td>
</tr>
<tr>
<td>2</td>
<td>647</td>
<td>83.6</td>
<td>12.92</td>
</tr>
<tr>
<td>Avg</td>
<td>639.1</td>
<td>87.8</td>
<td>13.71</td>
</tr>
</tbody>
</table>
Figure 1. Posthatch growth in trial I

Figure 2. Observation of leg deformation in trial I
Table 3. Number of eggs used and hatched in trial II (30 eggs)

<table>
<thead>
<tr>
<th></th>
<th>No injection: 10 eggs</th>
<th>Injection (antibody): 10 eggs</th>
<th>PBS injection 10 eggs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hatching</td>
<td>8</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>Failed hatching</td>
<td>2</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>posthatch Survival %</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Figure 3. Posthatch growth in trial II
Figure 4. Observation of leg deformation in trial II
Fertilized eggs were obtained from the Asagi Hatchery, INC. In ovo administration of 200 μl anti-prepro-myostatin antibody or PBS buffer was performed at 3 days after incubation through the blunt end of the eggs. Anti-prepro-myostatin antibody was diluted in PBS buffer at 120 ng / 200 μl. Prior to injection, the blunt end of egg was sterilized with 70% ethanol. A single hole was created with a micro drill without penetration into the chorio-allantoic membrane. Both antibody and PBS buffer were injected into the egg yolk on embryonic day 3 with a 22 gauge needle. The hole was sealed with parafilm, then the parafilm was melted by rapid heat application with torch. Eggs were set in incubator/hatcher (temperature 37 ± 0.5°C, humidity 86-87%) (Kocamis et al., 1998, 2000 and 2002) until hatching.

In trial I, only 2 chicks out of seven fertilized eggs were hatched after injection, and in trial II, the hatchability was improved: seven were hatched out of 10 eggs injected. The mean body weight of chicks injected with antibody in ovo was lower than that of chicks with no injection at around 20 day in both trial, but the statistical analyses could not be performed due to the small number of samples. The most striking difference observed in the chicks injected with antibody in ovo was a leg deformation as was shown in the picture. The leg deformation was observed in all treated chicks in both trials. The leg deformation was not observed in chicks injected with PBS in ovo, indicating that the leg deformation is probably caused by the anti-myostatin antibody not by the injection itself.

The polyclonal anti-prepro-myostatin appeared to bind to myostatin prodomain in our binding characterization of the antibodies. In this trials it was hypothesized that the anti-prepro-myostatin antibody binds to myostatin prodomain, thus suppress the
prodomain’s inhibitory effect of the biological activity of myostatin, resulting in stimulation of myostatin’s biological activity. If the above hypothesis is true, we expected that the chicks injected with antibodies in ovo will show diminished growth because of suppressed muscle development during embryonic stage. Interestingly, the mean body weight of chicks injected with the antibody was lower as we hypothesized. However, the number of chick used in those trials was too small to reach to any meaningful conclusion. Therefore, further studies are warranted to confirm the above preliminary results. In regard to the bone deformation, we have currently no explanation for this observation. Considering that bone morphogenic factors belong to the same family of proteins (TGF-β family) as myostain, and the family member proteins share homology in protein structure, it is speculated that potential reaction of the polyclonal anti-prepro-myostatin antibodies with the other TGF-β family member proteins might influence the bone formation during the embryonic development.