MYOSTATIN AND INHIBITION BY ITS PROPEPTIDE IN PREGNANCY AND OLD AGES

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAI‘I AT MĀNOA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

MOLECULAR BIOSCIENCES AND BIOENGINEERING

MAY 2013

BY

AWAT NOORADIN YOUSIF

Dissertation Committee:

Jinzeng Yang, Chairperson

Harry Ako

Yong Soo Kim

Yong Li

Douglas Vincent

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 - 1346

ACKNOWLEDGMENTS

First and foremost I must express sincere thanks to my advisor Dr. Jinzeng Yang, for his patient guidance and critical comments on my research, for sharing his expertise, knowledge during my research work at his lab and my study at the University of Hawaii. I would like to express my gratitude to all my dissertation committee members: Dr. Douglas Vincent, human nutrition, food and animal science department chair, Dr. Harry Ako, Dr. Yong Li. I must express my sincere thanks to Dr. Yong Soo Kim, for allowing me to use his lab, for his critical review and suggestions to my research proposals and lab work, for sharing his expertise and knowledge in the field of myostatin and muscle biology, and for serving in my dissertation committee.

I would also like to thank Dr. Dulal Borthakur, for accepting me in the MBBE program, for his continuous encouragement, fundamental help and support during my study journey at the University of Hawaii.

I would like to thank the financial and logistical support for part of my study which is provided by the Kurdistan Agricultural Higher Education and Development (KAHEAD) project, under the partnership agreement between the University of Hawaii at Manoa and the ministry of higher education and scientific research, Kurdistan Regional Government. I express my gratitude to Dr.Samir El-Swaify (KAHEAD Director, and Principal Investigator), Dr.Ekhlass Jarjees (KAHEAD Manager), Ms.Fanar Jarjees (KAHEAD Administrative Assistant), Ms. Patricia Brands (KAHEAD Librarian), and Dr.Adel Youkhana for their constant support and friendship.

I will not forget to express my appreciation to Dr. Linda Duckworth, Director, Martha Staff, Assistant Director, Danita Dumadag-Hugh, Secretary, and all other staffs at International Student Services (ISS) at the University of Hawaii at Manoa, for facilitating all my official papers, and for their financial support of part of my study throughout a very kind scholarship (Kenji Yamaguma Scholarship) which solved my unforeseen situation at the end of my study, thank you again.

I would like to thank Dr.Halina Zaleski and Ahmed Hussein for guiding me and performing statistical analysis for my experiments data, I also thank my lab colleagues,

Tuk Waraporn, and Benjamart Pratoomthai from Bangkok, Thailand. Vishal Singh Negi, Zicong Lee, and Gang Pan for their help and friendship.

I thank Dr.May Jaff, and Dr.Aram Abbas, Dean of faculty of agriculture back home at the University of Sulaimani-Kurdistan of Iraq for their continuous help and support by facilitating my official papers to travel to Hawaii.

Most of all, I am very grateful for my family, especially my mother, my father (*in memoriam*), my sisters, with their endless support, patience, praying and encouragement, for their financially support to the last part of my study. Throughout my life, they heavy instilled in me the determination required to complete this work. Above all, they have provided me with unending love and have lifted me up for the stars.

ABSTRACT

Skeletal muscle growth and maintenance are essential for human health, as well as have an economic importance for agricultural animals. A better understating of skeletal muscle growth is important by increasing understanding of physiological processes and molecular pathways associated with skeletal muscle growth and development. Myostatin or growth differentiation factor-8, as a member of transforming growth factor (TGF) superfamily, is a remarkable inhibitor for muscle growth, it can be inhibited by its own propeptide, myostatin propeptide is generated from the N-terminal peptide present in the myostatin precursor. Myostatin propeptide has been shown to promote muscle growth by blocking myostatin function. Myostatin is expressed primarily in the muscle tissues and to some degree in adipose tissues; it also has been detected in a number of other tissues. The increased birth weight and muscle growth in myostatin propeptide transgenic mice indicate that myostatin may be expressed in placenta tissue and inhibit fetal and placental growth. This project was designed to determine myostatin gene expression level in placenta tissue throughout different gestational stages, and its effect on fetal and placental growth, also effects of myostatin inhibition by its propeptide on muscle mass and fat accumulation in old ages. Three groups of mice were used, first group was wild type mice considered as control group, second group was heterozygous ($MSTN^{TG/+}$) transgenic mice, third group was homozygous ($MSTN^{TG/TG}$) transgenic mice. Placenta tissue samples and fetuses were collected and weighed at day 10 and 16 of the gestation. Total RNA was extracted from placenta samples for measuring gene expressions. The results showed that transgenic pregnant groups showed significant ($P<0.05$) higher live weight in comparison to their wild type littermates because of carrying significant larger

transgenic fetuses and placenta in their uterus, placenta myostatin mRNA showed (21.22±7.31) fold increase in heterozygous and (7.85±1.15) fold increase in homozygous pregnant groups at day 10 of gestation relatively compared to wild type group, while in day 16 the increased level was (12.28±4.97) and (4.98±0.52) fold in heterozygous and homozygous respectively. A positive correlation was found between myostatin expression level and gestational age in wild type group, while in both transgenic groups this correlation was negative.

To investigate effects of myostatin suppression by its propeptide on muscle mass and fat accumulation in old ages; we used four different ages of transgenic and wild type male mice: 1month (growing), 6 month (adult), 12 month (middle age), and 18 month (aged mice), whole body, individual muscles, carcass, white and brown adipose tissue weights were taken, total RNA were extracted from muscle tissues for later gene expression measurement. The results showed that the transgenic animals have more muscle mass and significantly ($P<0.05$) reduced fat accumulation by 80% compared to wild type littermates at old ages. The qRT-PCR results for gene expression showed myostatin mRNA level increased in transgenic mice and its level fluctuating between high and low during different time points of age. Each of MyoD and Myf5 mRNA level showed higher fold change in transgenic mice in early age of our experiment relatively compared to wild-type mice, while their levels reduced gradually with advancing in age. Pax7 gene showed higher expression in transgenic mice compared to wild-type groups at different ages; its level reached the highest expression in 18 months of age in transgenic mice.

The overall results of this study provide support for the role of myostatin in fetal and placental growth, also its role in skeletal muscle maintenance in old animals, and the prospect of targeting myostatin to prevent or reverse progressive muscle wasting that occurs in aging and certain degenerated diseases. Based on the above results myostatin inhibition can enhance and continue skeletal muscle growth in advanced age without accumulation or increase adipose tissue, this dramatic effect of myostatin on muscle mass in old ages is probably due to the ability of myostatin to negatively regulate skeletal muscle through myogenic regulatory factors and Pax7 gene.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

LITERATURE REVIEW

SKELETAL MUSCLE GROWTH AND DEVELOPMENT

1.1 Prenatal (embryonic) development of skeletal muscle

During early embryonic stage, muscle develops from mesoderm, one of the three germ layers (endoderm, mesoderm and ectoderm), skeletal muscle progenitor cells arise from the somites, which are formed sequentially as epithelial spheres from the rostral part of the unsegmented paraxial mesoderm (Stockdale et al., 2000). The somites mature according to a rostro-caudal gradient of differentiation and become subsequently subdivided into the ventrolateral sclerotome that gives rise to axial skeleton and ribs, and the dorsomedial dermomyotome that contributes to the formation of dermis and skeletal muscles (Stockdale et al., 2000). Two distinctive compartments present in the dermomyotome give rise to separate lineage of skeletal muscles during embryogeneis: the 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. 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 wings and limbs (Ordahl and Ledouarin, 1992; Miller et al., 1999).

The myotomal compartments of somites eventually give rise to myoblasts, mono-nucleated muscle precursor cells. During embryonic muscle development, the mono-nucleated myoblasts fuse together to form multi-nucleated myotubes. Later, myotubes mature into highly specialized skeletal muscle fibers that show cross striation. The determination of myogenic cell lineage (myoblast) and control of terminal differentiation is regulated by the expression of myogenic regulatory transcription factors (Perry and Rudnicki, 2000). In addition, the presence of some particular growth factors such as insulin, insulin like growth factor-1 (IGF-1), fibroblast growth factor (FGF) and

transforming growth factor (TGF)-β, regulates sustained proliferation of myoblasts and subsequent withdrawal from the cell cycle as part of differentiation process (Florini et al., 1996). The timing of entry of myoblasts into differentiation is likely to play a significant role in determining the number of muscle fibers, and subsequent postnatal muscle mass.

1.2 Myogenic regulatory factors (MRFs)

Myogenic regulatory factors (MRFs), form a family of basic-Helix-Loop-Helix (bHLH) transcription factors, including MyoD, Myf5, myogenin and MRF4, which are a group of transcription factors that contain a conserved E-box DNA- binding domain (Weintraub et al., 1991; Perry and Rudnick, 2000). Expressions of these myogenic transcription factors induce differentiation of muscle stem cells into myoblasts. Cells producing a myogenic transcription factor are committed to becoming muscle cells, since transfection of genes encoding any of these myogenic proteins into various cultured cells convert those cells into myoblasts (Thayer et al. 1989; Weintraub et al. 1989).

Studies have shown that there is a strong relationship between myogenic regulatory factors by gene targeting study. It has been shown redundancy function between them (Megeney and Rudnicki, 1995). Basically MRFs can be divided into two groups depending on their functions; MyoD and Myf5 considered as the primary MRFs, they are required for the determination of skeletal myoblasts. Myogenin and MRF4 considered as secondary MRFs because they act later in the development as differentiation factors. The primary MRFs are expressed in proliferating myoblasts before differentiation, and the secondary MRFs are expressed in terminally differentiating cells.

MyoD knockout mice 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. Myogenin knockout mice 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. Deletion mutation in MRF4 shows normal Myf-5 expression and a four-fold increase in myogenin, and is viable with seemingly normal skeletal muscle (Zhang et al., 1995). Myf-5 and MRF4 knockout mice 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).

1.3 Postnatal development of skeletal muscle

1.3.1 Muscle fiber number

Fusion of multiple myoblasts produce muscle fiber and numerous muscle fibers together produce muscle. Muscle growth is determined by an increase in muscle fiber number (hyperplasia) as well as an increase in muscle fiber size (hypertrophy). The number of muscle fiber usually is fixed before birth (Luff and Goldspink, 1970; Wegner et al., 2000). Postnatal muscle growth mainly results from muscle hypertrophy. Muscle fiber enlargement could be the consequence of increase in activity of satellite cells, which fuse to adjacent muscle fibers to increase their size. After birth, the number of skeletal muscle fiber in most mammals and avian species does not increase due to the completion of embryonic proliferation of skeletal muscle cells (Rowe and Goldspink, 1969). No significant changes in postnatal fiber number have been found in mice (Rowe and Goldspink, 1969), rat (Rosenblatt and Woods, 1992), pig (Fiedler et al., 1983), cattle (Wegner et al., 2000) and chicken (Smith and Fletcher, 1988). However, some studies indicated that in some species, limited extent of muscle cell proliferation occurs after birth. For example, the increase in muscle fiber number was observed shortly after birth

in rodents (Summers and Medrano, 1994) and pigs (Swatland, 1975). Others argued that the increase in fiber number during the first days of postnatal life was a result of maturation and elongation of the existing myotubes rather than due to a production of new fibers (Ontell and Kozeka, 1984). The postnatal increase in skeletal muscle mass appears to be achieved mostly by an increase in fiber size and not much by an increase in fiber number. 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 (Powell and Aberle, 1981). Interestingly, some studies indicate that muscle hypertrophy is inversely correlated with muscle fiber number at the end of the intensive growth period. For example, the postnatal growth rate of the individual muscle fiber is lower when there are high numbers of fibers and higher when there are low numbers of fibers (Rehfeldt et al., 2000).

1.3.2 Muscle fiber types

Based on their biochemical and physiological properties, muscle fibers can be generally divided into three types, including Type 1 fibers (also called Slow-twitch oxidative fibers or Red fibers), Type ΠA fibers (also known as Fast-twitch oxidative glycolytic fibers or Intermediate fibers) and Type ΠB fibers (also referred to as Fast-twitch glycolytic fibers or White fibers) (Gauthier, 1969; Brooke and Kaiser, 1970; Peter et al., 1972). Individual muscles are a mixture of 3 types of muscle fibers, but their proportions depend on the function of that muscle. Type 1 fibers are characterized by a slow contraction time and a high resistance to fatigue. Structurally, they have high mitochondrial and capillary density, and high myoglobin content that is responsible for their red color. These fibers use oxidative metabolism to generate ATP for contraction. Muscles mainly containing Type 1 fibers are used for aerobic activities requiring low-level force production, such as walking and maintaining posture. Most daily living activities use these muscles. Type ΠB fibers are identified by a quick contraction time

and a low resistance to fatigue. They contain a low mitochondrial and capillary density and myoglobin content. They mainly rely on anaerobic glycolysis to generate energy for contraction. Functionally, Type ΠB fibers-dominant muscles are used for anaerobic activities with a high force output, such as sprinting and jumping. Type ΠA fibers represent a transition between Type 1 and Type ΠB fibers. They have the characters of both Type 1 and Type ΠB fibers. Since Type Π fibers (Including Type ΠA and Type ΠB fibers) have a higher glycogen concentration and are metabolically better equipped than type I fibers for post-mortem anaerobic glycolysis, which causes changes in color and impairments in water holding capacity of fresh meat, leading to development of pale, soft, and exudative (PSE) meat (Klont et al., 1998). Therefore, a high ratio of Type 1 fibers to Type Π fibers in muscles is good for meat quality.

1.3.3 Satellite cells

Satellite cells are undifferentiated muscle precursor cells lying between the basal lamina and the muscle fibers, which are responsible for postnatal growth, repair, and maintenance of skeletal muscle (Seale and Rudnicki, 2000). Postnatal muscle growth mainly results from muscle hypertrophy. Muscle fiber enlargement could be the consequence of increase in activity of satellite cells, which fuse to adjacent muscle fibers to increase their size. Satellite cells have one nucleus that occupies most of the cell volume and unlike the nuclei inside muscle fibers, the satellite cells retain the capacity to proliferate in response to stimuli, thus provide most of the myonuclei to adult muscles during growth to balance the cytoplasm/nuclei ratio. Satellite cells have long been considered monopotential only giving rise to cells of myogenic lineage (Bischoff and Heintz, 1994). However, recent evidence demonstrates that satellite cells are capable of forming osteocytes and adipocytes in response to different growth factors *in vitro*, indicating that satellites possess multipotential mesenchymal stem cell activity (Asakura et al., 2001).

The expression of MRF 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).

Stretch-overload could induce hypertrophy in skeletal muscle whose satellite cells were inactivated by irradiation (Lowe and Alway, 1999), supporting that satellite cell activation is not prerequisite for muscle hypertrophy. In contrast, (Kawano et al., 2008) reported that satellite cell activation in response to mechanical load/or neural activity plays an essential role in muscle hypertrophy. While the debate on the role of satellite cells on muscle hypertrophy is not settled, it is generally agreed that experimental models (type of hypertrophic stimuli) and situations (magnitude of growth response and the age of animal, timing of measurement) influence the relationship between muscle hypertrophy and satellite cell activation (O'Connor et al., 2007). As animals age, however, the satellite cell population decreases in absolute number and as a percentage of nuclei in skeletal muscle tissue (Snow, 1977). The distribution of satellite cells also varies among muscle fiber types with slow muscle fibers having a greater proportion than fast muscle fibers (Chen and Goldhamer, 2003). Satellite cells, in a similar manner to embryonic myoblasts, undergo multiple rounds of division prior to terminal differentiation and fusion to form multinucleated myofibers by mechanisms which are not clear (McKinnell et al., 2005). Unlike myoblasts, however, satellite cells maintain their ability to self-renew by generating identical daughter cells to restore the pool of quiescent satellite cells in a process called asymmetric division (Kuang et al., 2007).

Myostatin is expressed in satellite cells and regulates satellite cell quiescence and self-renewal (Cornelison et al., 2000). Higher levels of myostatin have been observed in quiescent satellite cells and lower levels are observed when satellite cells are activated (McCroskery et al., 2005). Also, higher levels of myostatin are observed during immobilization, resulting in suppression of satellite cell activation (Carlson et al., 1999). In vitro studies revealed that myostatin null mice also exhibit greater proliferation and earlier differentiation of satellite cells (Wagner et al., 2005).

1.4 Transforming growth factor β superfamily

The transforming growth factor β (TGF-β) superfamily is a group of secreted growth and differentiation factors (GDF) which maintain tissue homeostatis (McPherron and Lee, 1997). The members of this superfamily are involved in a variety of biological functions including development, differentiation, reproduction, immune responses, bone growth, apoptosis, tissue homeostasis and regeneration (Kolodziejczyk and Hall, 1996; McPherron et al., 1997; Hoodless and Wrana, 1998; Schulz et al., 2008).

1.4.1 Myostatin

Myostatin is a member of the TGF-β superfamily of growth factors, which regulates tissue growth, also known as growth differentiation factor 8 (GDF-8), was first identified by (McPherron et al. 1997) in a study seeking novel mammalian members of the transforming growth factor-β (TGF-β) superfamily. The study reported that knock-out of the myostatin gene increased muscle mass up to three-fold in mice. Hypermuscularity caused by natural mutations on the myostatin gene has been identified in several mammalian species, including cattle (Kambadur et al., 1997; McPherron and Lee, 1997), dogs (Shelton and Engvall, 2007), sheep (Hadjipavlou et al., 2008), and human (Schuelke et al., 2004).

Myostatin is synthesized as a precursor protein of about 52 kDa with 375 amino acids (376 amino acids in rodents) encoded from a single open reading frame that acts to inhibit muscle growth. Like other members of the TGF-β superfamily, Myostatin precursor protein contains an N-terminal signal sequence for secretion, a dibasic proteolytic processing site, and a carboxy-terminal (C-terminal) region with nine cysteine residues, one of which contributes to the formation of a disulphide-linked Myostatin dimer with a size of 25 kDa soon after translation (McPherron et al., 1997). It is assumed that the N-terminal propeptide aids in proper folding of the C-terminal region of myostatin into cysteine knot structure (Thomas et al., 2000; Jin et al., 2004). The amino acid sequence of the C-terminal of myostatin is highly conserved across species like mouse, human, cattle, pig, dog, chicken, fish, and mollusc, which suggests a potentially

common and conserved function of Myostatin on inhibiting skeletal muscle growth (McPherron et al., 1997; Hu et al., 2010b). Like other members of the TGF- β superfamily, the Myostatin homodimer is regarded as the biologically active form and possesses receptor-binding activity as shown in vitro studies (Lee and McPherron, 2001; Thies et al., 2001; Rebbapragada et al., 2003). However, due to the similarity with other members of the TGF- β superfamily, we cannot rule out the possibility that Myostatin might form heterodimers with the other family members as well.

Myostatin undergoes two proteolytic processing events in order to generate the biologically active molecule. The first cleavage event removes the signal peptide, which plays a role in the transport of myostatin precursor protein from the cytoplasm into endoplasmic reticulum. The second cleavage event generating N-terminal propeptide and C-terminal active-form of myostatin occur at the tetrabasic (RSRR) site, which is mediated by a calcium-dependent serine protease called furin (Lee and McPherron, 2001; Thies et al., 2001). After the proteolytic processing of the precursor, Myostatin is secreted as an inactive latent complex of a mature dimmer bound by two propeptides, thus the propeptide is functioning to suppress myostatin activity as it is shown in figure 1.1. In support of the role of propeptide to inhibit myostatin biological activity, the over-expression of myostatin propeptide in mice resulted in a dramatic increase in skeletal muscle mass (Lee and McPherron, 2001; Yang et al., 2001). In addition, the administration of recombinant myostatin propeptide (Li et al., 2010) or plasmid- or adeno-associated virus-mediated delivary of myostatin propeptide induced an increase in muscle mass (Matsakas et al., 2009; Hu et al., 2010), as can be seen in figure 1.2.

Binding of mature Myostatin to its receptor (activin type IIβ receptor) for induction of intracellular signaling cascade, thus, requires dissociation of the propeptide from the latent complex. The propeptide binding to the mature region can be physically or biochemically destabilized after it is cleaved by protease, such as bone morphogenetic protein-1/tolloid (BMP-1/TLD) family of metalloproteinases (Wolfman et al., 2003; Lee, 2008).

Some of the TGF-β superfamily members, such as TGF-β 1, 2 and 3, are synthesized as precursors containing N-terminal propeptides that can form a latent complex with their own C-terminal mature proteins to inactivate their activities, following the proteolysis by

furin-like convertases (Böttinger et al., 1996). Like the propeptides of TGF-β 1, 2 and 3, myostatin propeptide, which is generated from the N-terminal propeptide of the myostatin precursor after proteolytic processing, also was shown to form a latent complex with mature myostatin both in vitro and in vivo (Lee and McPherron, 2001; Thies et al., 2001; Zimmers et al., 2002). In addition, the mouse myostatin propeptide was demonstrated to enhance muscle growth in vivo by suppressing myostatin function (Lee and McPherron, 2001; Yang et al., 2001), and its own activity is inhibited by BMP-1/TLD-like metalloproteinases (Pirottin et al., 2005; Wolfman et al., 2003).

In addition to the propeptide, several other proteins have also been shown to be capable of binding and inhibiting the activity of mature Myostatin. Several studies suggested that follistatin can function as a potent myostatin antagonist and plays an important role in modulating myostatin activity. Follistatin was capable of blocking myostatin activity in both receptor binding and reporter gene assays in CHO cells (Zimmers et al., 2002).

In mice, over-expression of follistatin in muscle induced dramatic increases in skeletal muscle growth (Lee and McPherron, 2001). Another study (Amthor et al., 2004) also showed that follistatin and myostatin interact directly with high affinity during chick development, and myostatin inhibits terminal differential of muscle cells in high-density cell cultures of limb mesenchyme and that follistatin rescues muscle differentiation in a concentration dependent manner. The results suggest that follistatin antagonizes myostatin by direct interaction, resulting in prevention of myostatin from executing its inhibitory effect on muscle development. Recently, gene-targeted approach of follistatin over-expression, such as adenoviral vector-mediated gene therapy and cell-mediated therapy using myogenic stem cells, showed that the over-expression of follistatin enhanced skeletal muscle growth in dystrophic mice (Nakatani et al., 2008; Tsuchida, 2008; Rodino-Klapac et al., 2009).

Mature myostatin function also can be regulating by Follistatin related gene (FLRG) and growth and differentiation factor (GDF)-associated serum protein-1 (GASP-1) (Hill et al., 2002; Hill et al., 2003; Takehara-Kasamatsu et al., 2007; Saremi et al., 2010). Hill et al. (2002, 2003) demonstrated that both FLRG and GASP-1 is complex to myostatin in the blood of mice and human, and studies with recombinant proteins have shown that

both proteins can bind with high affinity to the myostatin and inhibit its activity as assessed by reporter gene assays. Moreover, (Takehara-Kasamatsu et al., 2007) demonstrated that the over-expression of FLRG mRNA suppressed the Myostatin activity as a negative regulator during fetal and adult mouse heart development. In addition, Saremi et al. (2010) demonstrated that resistance training caused a significant decrease in serum levels of myostatin and increase in GASP-1. The effect of resistance training on serum levels of myostatin and GASP-1, may explain the increased muscle mass.

Titin-cap (T-cap), decorin and perlecan are other molecules which may involve in regulating the activity of Myostatin (Nicholas et al., 2002; Miura et al., 2006; Nishimura et al., 2007; Kishioka et al., 2008; Xu et al., 2010). The over-expression of titin-cap, a sarcomeric protein, in C2C12 myoblasts induced an increase in the rate of cell proliferation through suppression of myostatin expression, indicating that titin-cap may control myostatin secretion in myogenic precursor cells without affecting the processing step of precursor myostatin (Nicholas et al., 2002). Decorin, which is a small leucine-rich proteoglycan, binds to myostatin and blocks the inhibitory effect of Myostatin on muscle cell growth *in vitro* (Miura et al., 2006). Also, the over-expression of decorin enhances the proliferation and differentiation of C2C12 myoblasts through suppressing myostatin activity (Kishioka et al., 2008). In addtion, skeletal muscle-specific knock-out of perlecan, a component of the basement membrane that surrounds skeletal muscle, induced a decrease in myostatin expression and increased fiber cross-sectional area as compared with control mice, suggesting that perlecan is critical for maintaining skeletal muscle mass and fiber composition, and for regulating myostatin signaling (Xu et al., 2010).

Myostatin mRNA is mostly expressed in developing skeletal muscle of embryo and adult animals; it has also been reported to be expressed in other tissues including myoblast and fibroblast in regenerating skeletal muscle (Yamanouchi et al., 2000), cardiomyocytes and purkinje fibers of heart (Sharma et al., 1999; Shyu et al., 2005), spleen (Lyons et al., 2010), extraocular muscle (Toniolo et al., 2005; Patruno et al., 2008), mammary glands (Ji et al., 1998; Hosoyama et al., 2006), brain (Rodgers and Garikipati 2008), endometrium (Stoikos et al., 2008) uterus (Wong et al., 2009), skin (Zhang et al., 2012) human placenta (Mitchell et al., 2001) and, to a lesser extent, in adipose tissue (McPherron et al., 1997). In contrast with mammals, fish expresses myostatin mRNA in

muscles, eyes, gill filaments, spleen, ovaries, gut, brain and, at a lower level, in testes (Maccatrozzo et al., 2001; Rodgers and Weber, 2001).

1.4.2 Myostatin signaling pathway

After proteolytic cleavage of propeptide from the latent complex in the extracellular matrix (ECM), the mature myostatin dimer binds to either activin receptor type II A (ActRIIA) or ActRIIB, a family of serine/threonine kinase trans-membrane receptors expressed in bone marrow-derived mesenchymal stem cells (Hamrick et al., 2007) and located on the surface of the target cells (Rebbapragada et al., 2003). An in vitro study reported that myostatin dimer has a higher affinity to ActRIIB (Lee and McPherron, 2001). The importance of ActRII receptors in regulating myostatin signaling in vivo was demonstrated in transgenic mice, which expressed a dominant-negative form of ActRIIB without the kinase domain specifically in muscle using a myosin light chain promoter and enhancer (Lee and McPherron, 2001). These transgenic mice showed significant increases in muscle mass through both hypertrophy and hyperplasia, which were comparable to the effect of myostatin knockout in mice (Lee and McPherron, 2001). Upon binding of the ligand to the ActRIIB receptor, the activin type I receptor is phosphorylated and activated, which then initiates the intracellular signaling cascade through phosphorylation of the Smad2 and Smad3 proteins (Lee and McPherron, 2001). After oligomerization with a Co-Smad protein (Smad4), the Smad complex translocates from the cytoplasm into the nucleus, where it regulates the transcription of target genes like MyoD (Langley et al., 2002; Shi and Massague, 2003).

Myostatin dimer can also bind to either ALK-5 or ALK-4 type I receptors (Rebbapragada et al., 2003). ALK-4 is capable to interact with ActRII receptors whereas ALK-5 has only shown interaction with TGF-β RII receptors (Massague, 1998). This myostatin signaling pathway via ALK type 1 receptors continues by activating the cascade of Smad proteins and translocating into the nucleus to control gene transcription (Liu et al., 2001).

1.4.3 Myostatin and muscle growth

Myostatin negatively regulates skeletal muscle mass. Animals without functional myostatin exhibit primarily hyperplasia (an increase in the number of muscle fibers) and to a lesser extent hypertrophy (an increase in the size of muscle fibers) (McPherron & Lee, 1997). Studies have demonstrated that myostatin suppresses muscle growth in two ways: one way to limit muscle growth is by regulating the number of muscle fiber during development, and the other way is by regulating postnatal hypertrophy of skeletal muscle fibers. During embryogenesis, myostatin seems act to inhibit myoblast proliferation and differentiation *via* preventing cell cycle progression and down-regulating the level of myogenic regulatory factors, such as MyoD and myogenin (Thomas et al., 2000; Taylor et al., 2001; Langley et al., 2002; Rios et al., 2002). Suppression of myoblast proliferation by myostatin is believed to take place through down-regulating the activity of cyclin D1 at mRNA level as well as protein level, which causes cell cycle arrest at G1 phase (Yang et al., 2006; Ji et al., 2008). After birth, myostatin appears to limit skeletal muscle mass by depressing satellite cell proliferation (McCroskery et al., 2003), and inhibiting muscle protein synthesis in combination with activating muscle protein degradation (McFarlane et al., 2006). Inhibition of satellite cell division by myostatin is through decreasing CDK2 protein level in conjunction with up-regulating the activity of CDK inhibitor, p21 (McCroskery et al., 2003). However, some recent studies indicated that activation of satellite cells is not involved in postnatal muscle hypertrophy driven by myostatin blockade, since myostatin suppression-induced hypertrophic muscles contain no more myonuclei or satellite cells than controls (Amthor et al., 2009; Sartori et al., 2009). Myostatin had no significant effect on satellite cell proliferation in vitro, which is believed to be caused by the down-regulation of myostatin receptors in postnatal satellite cells (Amthor et al., 2009). These findings challenge the generally accepted model of myostatin-based regulation of postnatal muscle growth and suggest that postnatal muscle hypertrophy induced by myostatin blockade probably mainly results from increase in synthesis and turnover of muscle fiber structural proteins rather than enhancement of satellite cell proliferation. Myostatin inhibits Akt phosphorylation, which is required for activating the major muscle hypertrophy-stimulating pathway induced by IGF-1, to

depress muscle protein synthesis (McFarlane et al., 2006). Activation of muscle protein degradation by myostatin is thought to occur by increasing the expressions of two genes, atrogin-1 and muscle RING-finger 1 (MuRF1), which promote muscle protein breakdown primarily through the ubiquitin-proteasome proteolytic pathway (McFarlane et al., 2006; Satori et al., 2009). These findings clearly indicate that myostatin not only inhibits prenatal increase in muscle fiber number by suppressing myoblast proliferation and differentiation, but also inhibits postnatal increase in muscle fiber size through depressing muscle protein turnover. It, therefore, appears that blocking myostatin function may offer a strategy for enhancing muscle growth in agriculture animals as well as for treating muscular disorders in humans.

1.4.4 Myostatin and fat accumulation

Myostatin is expressed primarily in the skeletal muscle and to some degree in adipose tissue. Mice with a mutation in the myostatin gene have a significant increase in skeletal muscle mass throughout their body (McPherron et al., 2002). Diabetes mellitus is characterized by insulin resistance and disrupted glucose absorption in skeletal muscle. Since myostatin triggers increased muscle mass and has shown the ability to slow or prevent fat accumulation, it has been a target for possible pharmacological treatment of diabetes (Tsuchida, 2004). McPherron and Lee, 2002, showed that loss of myostatin function prevents age-related adipose tissue accumulation and positively affects serum glucose and insulin levels. The myostatin mutation was also able to partially attenuate obese and diabetic phenotypes of two mouse models, Lep ob/ob and KKAy, used to study obesity and diabetes. There are two theories presented in their experiment to explain how loss of myostatin affects fat deposition and accumulation. One of their theory is that myostatin has a direct effect on adipose tissue. The second theory is that the myostatin mutation has an indirect effect by causing an increase in skeletal muscle mass therefore utilizing more fuel. The secondary effect is that less energy is available to be stored as fat. The other explanation for an indirect effect of myostatin is that the lack of myostatin in muscle affects the activity of a theoretical second messenger (McPherron et al., 2002).

Myostatin has been shown to have an inhibitory effect on differentiation of pre-adipocytes (Kim et al., 2001). One proposed mechanism for its inhibitory effect is by inhibition of PPAR-γ and CCAAT/enhancer binding proteins (C/EBP), which are key transcription factors necessary for adipocyte differentiation. Kim et al, 2001, demonstrated that treatment of 3T3-Ll (mouse pre-adipocytes) cells with myostatin inhibited adipocyte differentiation in a dose-dependent manner. Expression of PPAR-γ and C/EBP-α in 3T3-Ll cells were significantly reduced when myostatin was added to the cell cultures (Kim et al., 2001). This led to the conclusion that the mechanism by which myostatin inhibited differentiation in pre-adipocytes was by decreasing the expression levels of PPAR-γ and C/EBP-α. However, conflicting reports on the action of myostatin on pre-adipocytes exist. Another study by Artaza et al, 2005 demonstrated that recombinant myostatin promoted the differentiation of the multipotent C3H 10T(1/2) cells into adipogenic cells by up-regulating C/EBP-α in a dose-dependent manner (Artaza et al. 2005). In support of this study, Lin et al, 2002, reported that myostatin knockout mice had significantly lower fat pad weights, increased muscle mass, and decreased circulating levels of PPAR-γ and C/EBP-α (Lin et al. 2002). Authors theorized that the mechanism by which myostatin knock-out mice decreased fat accumulation was by decreasing adipogenesis which was reflected in the decrease in concentration of adipogenic factors PPAR-γ and C/EBP-α. In conclusion, the mechanism by which myostatin affects adipogenesis and differentiation of pre-adipocytes remains unclear and is still under investigation.

1.4.5 Myostatin and the age-related loss of skeletal muscle mass (Sarcopenia)

Aging has been associated with a loss of muscle mass that is referred to as ‘sarcopenia’. Sarcopenia is the degenerative loss of skeletal muscle mass and strength associated with aging. Loss of muscle mass among the aged directly results in diminished muscle function. Sarcopenia is also associated with an increased incidence of metabolic disorders, including obesity and diabetes (Roubenoff et al., 1998), and there is a critical need for treatments that attenuate sarcopenia. Although exercise training has been shown to attenuate sarcopenia, it cannot prevent or reverse it completely (Faulkner et al., 2007).

Furthermore, the aged often suffer from other debilitating conditions such as osteoarthritis and immobility, which may preclude physical exercise as a viable treatment for sarcopenia. As a consequence, there is a need for pharmacological interventions that may be combined with exercise training regimes to treat sarcopenia effectively (Lynch, 2004). Myostatin as a negative regulator of muscle mass has shown to involve in muscle atrophy and increased protein degradation. For instance, patients with HIV express higher levels of myostatin in conditions of muscle wasting (Gonzalez-Cadavid et al., 1998). Additionally, cachexia is induced in animals over-expressing myostatin (Zimmers et al., 2002). Conversely, administration of a myostatin antibody to dystrophic mice increases body and muscle weights while simultaneously reducing muscle degeneration and increasing levels of creatine kinase (Bogdanovich et al., 2002). Myostatin has been shown to induce atrophy by up-regulating genes involved in ubiquitin-mediated proteolysis (McFarlane et al., 2006).

Studies have shown that reducing myostatin activity results in increased muscle mass in several disease models, including muscular dystrophy (Wagner et al., 2002; Bogdanovich et al., 2002; Bogdanovich et al., 2005) and cancer cachexia (Liu et al., 2008). Studies in wild-type and Mstn-/- mice found that the prolonged absence of myostatin attenuated age-related decreases in muscle fiber size and satellite cell activation (Siriett et al., 2006). However, it is difficult to determine the clinical relevance of results obtained in transgenic mice with lifelong inhibition of myostatin. Furthermore, treatment with a myostatin antagonist (Mstn-ant1) for 6 wk enhanced grip strength and satellite cell activation in middle-aged (13–16 month) mice (Siriett et al., 2007). Only limited data are available in muscles from older mice (24 month) that exhibit an age-related decrease in muscle mass and function (LeBrasseur et al., 2009). Four weeks of treatment with a myostatin inhibitory antibody (PF-354) enhanced muscle mass (by 12%) and reduced fatigability, but the dose of PF-354 used in that study was impractically high (24 mg/kg) for clinical translation, and we have previously found similar improvements in muscle mass with a more clinically relevant dose of PF-354 (10 mg/kg) (Murphy et al., 2010).

The findings obtained in 13–16 month-old mice and in Mstn-/- mice support the traditional belief that the primary effect of inhibiting myostatin is satellite cell activation

through inhibition of the intracellular Smad signaling pathway (McCroskery et al., 2003). Myostatin blockade enhances muscle protein synthesis (Welle et al., 2009) by potentially relieving the inhibition normally imposed on the Akt/mTOR signaling pathway by myostatin (Amirouche et al., 2009), although this mechanism remains controversial (Welle et al., 2009). Myostatin blockade may also attenuate muscle protein degradation. Although the mechanisms mediating this effect have not been well studied, they could include inhibition of the ubiquitin-proteasome system, which is controlled, in part, by Akt (LeBrasseur et al., 2009; McFarlane et al., 2006). Another potential mechanism includes attenuation of apoptosis, with microarray analysis of skeletal muscles from Mstn-/- mice showing increased expression of antiapoptotic genes compared with control mice (Chelh et al., 2009). Recent studies have highlighted the importance of apoptosis in sarcopenia (Dirks and Leeuwenburgh, 2002; Siu, 2009) and have implicated both the (intrinsic) mitochondrial and (extrinsic) death receptor apoptotic pathways in sarcopenia, although the mitochondrial pathway appears to precede initiation of the extrinsic pathway (Siu, 2009). The extent of sarcopenia due to apoptosis has not been fully elucidated, but muscles from aged animals exhibit more than 50% increase in the expression of proapoptotic markers (Siu, 2009), indicating that the contribution of apoptosis to sarcopenia is significant. Myostatin inhibition could, therefore, attenuate sarcopenia by reducing apoptosis, but this has yet to be investigated. Blocking or inhibiting myostatin may have practical implications for preventing muscle loss. Thus this novel gene could potentially affect both the livestock and biomedical industry.

1.5 Quantitative real-time PCR (qRT-PCR)

qRT-PCR is a laboratory technique based on the PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule. For one or more specific sequences in a DNA sample, qRT-PCR enables both detection and quantification. The quantity can be either an absolute number of copies or a relative amount when normalized to DNA input or additional normalizing genes. Unlike conventional PCR, qRT-PCR collects data as the PCR process occurs, rather than at the endpoint of the reaction. Researchers use qRT-PCR because it quantifies differences in mRNA expression but uses smaller amounts of RNA than Northern blotting (Bustin, 2002). This is advantageous when experiments

generate only small amounts of RNA such as in cell culture experiments or with samples from small animals.

There are two methods which can be used as procedure for qRT-PCR; one-step or two-step approach. In the one-step approach, reverse transcription and PCR are both performed in the same reaction tube. In the two step approach, reverse transcription is performed prior to qRT-PCR. The one-step approach is quicker, less expensive, and requires less handling of samples reducing pipeting errors and possible contamination. Gene specific primers are used during reverse transcription, however, limit quantification to one gene at a time. The two-step approach uses random hexamer or oligonucleotide-dt primers to amplify all the mRNA present allowing for multiple genes to be investigated simultaneously (Wong & Medrano, 2005). Also, two-step converts RNA into cDNA which is easier to store and less susceptible to degradation than RNA. Studies have shown that while the two approaches are similar in efficiency, the two-step approach may be slightly more sensitive and able to detect genes expressed in lower quantities (Peters et al., 2004).

Release of a fluorescent molecule is measured during real time reactions using Applied Biosytems Taqman Primer/Probe system, (Heid et al., 1996). Probes contain two fluorescent dyes; one which serves as a reporter at the 5' end of the primer and one that serves as a quencher on the 3'end. The close position of the quencher dye greatly reduces the fluorescence of the reporter dye (by fluorescence resonance energy transfer). When the probe anneals to a target sequence, it is cleaved by the 5' exonuclease activity of Taq DNA Polymerase as the primer is extended (Holland et al., 1991). Upon cleavage, the reporter dye emission is no longer transferred to the quencher dye, thus, fluorescence only occurs when new DNA is synthesized from the cDNA template.

The number of threshold cycle (Ct) is the point at which the fluorescence crosses a threshold. Fewer cycles are needed to produce a detectable signal when a greater amount of target molecules are present. Therefore a lower Ct value means more mRNA of a target sequence was present while a higher Ct values corresponds to less mRNA produced for a target sequence (Higuchi et al., 1993). The Ct values are then standardized to the Ct of a housekeeper gene, a gene whose expression is stable among treatments. This standardizes between samples for differences in starting cDNA concentrations.

Mathematical calculations allow for comparisons between groups or treatments in a fold-change manner. A fold change equal to one indicates no differences in mRNA expression between treatments. Fold changes greater than one indicate up-regulation of mRNA, and fold changes less than one indicate down-regulation of specific mRNA.

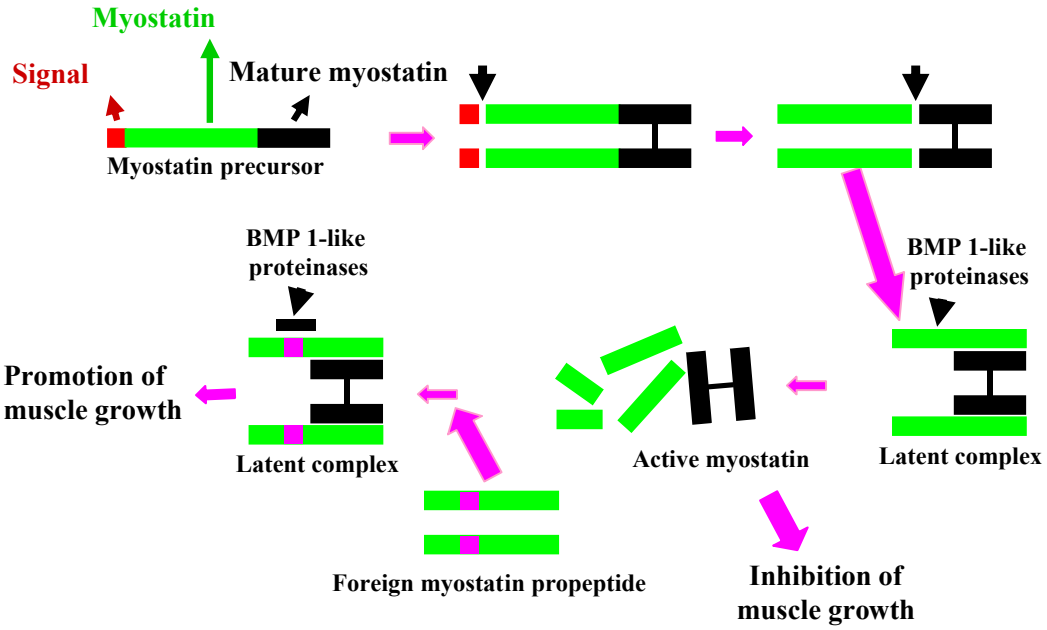


Figure 1.1 The mechanism for myostatin propeptide to inhibit myostatin activity

Figure 1.2 Phenotype of transgenic myostatin propeptide over-expression (left) and littermate control mice (right).

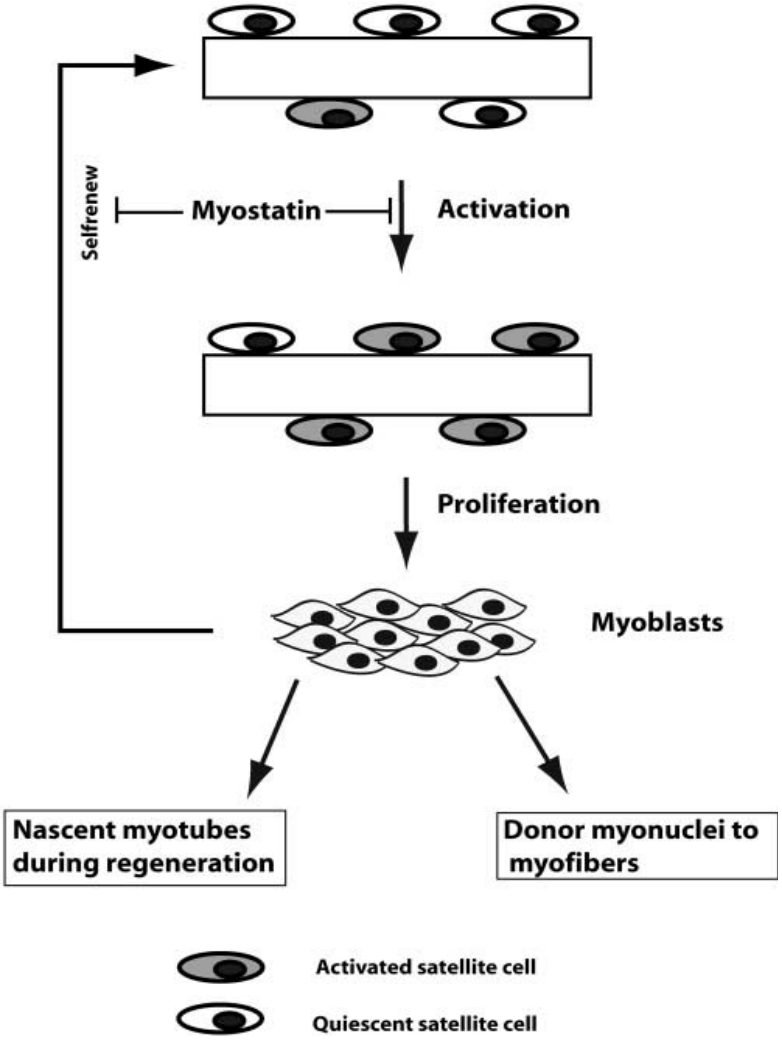


Figure 1.3 A model for the role of Myostatin in postnatal muscle growth. Quiescent satellite cells on muscle fibers are activated in response to muscle injury to give rise to myoblasts. Proliferating myoblasts can either fuse with the existing fiber or differentiate into a nascent myotube. A portion of proliferating myoblasts, however, can revert to become quiescent satellite cells, thus resulting in self-renewal. As myostatin is a negative regulator of cell cycle progression, high levels of myostatin in satellite cells block the activation to maintain quiescence by (McCroskery et al., 2003)

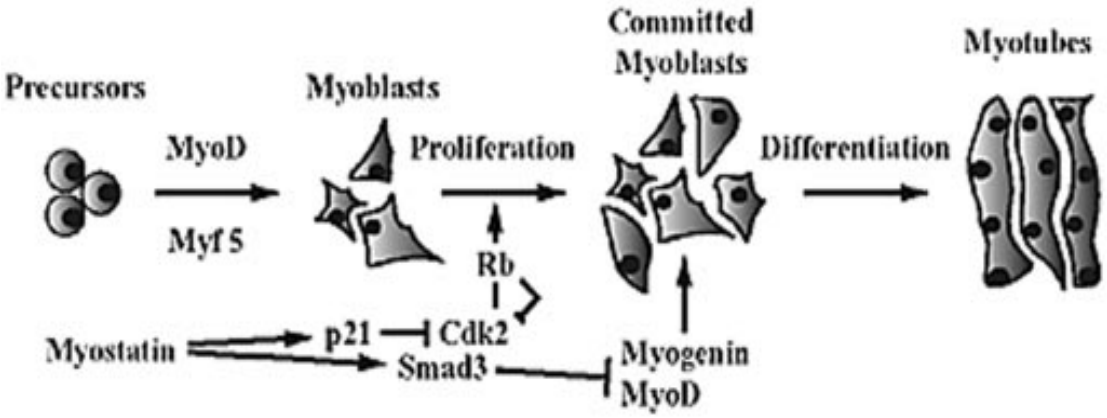


Figure 1.4 A model for the role of myostatin during muscle growth and differentiation. A model from (Thomas et al., 2000) for the role of myostatin during muscle growth, adapted to include the role of myostatin during myogenic differentiation. During myogenic embryogenesis, Myf5 and MyoD specify cells to adopt the myogenic fate. Myoblast proliferation is regulated by myostatin via the up-regulation of p21 and inactivation of Cdk activity resulting in retinoblastoma (Rb) hypophosphorylation and myoblast cell cycle arrest. In response to a differentiation cue, MyoD becomes fully functional, activating downstream myogenic gene expression, including myogenin and p21, resulting in committed myoblasts that fuse into multinucleated myotubes. Myostatin regulates this process by inhibiting MyoD expression via Smad 3 after the differentiation cue resulting in the loss of downstream myogenic gene expression and myogenic differentiation. By (Langley et al., 2002)

1.6 Reproduction in mice

Mouse (Mus musculus) has many advantages as research animals, they are easy to care for and handle, and are relatively inexpensive compared to other species. A high reproductive performance with a large litter size and a short gestation means that many generations can be produced in a relatively short period of time. Litter sizes average 6-12 pups, though it is not unusual for a female's first litter to be smaller in number. Litter sizes decrease as breeding female's age. Sexual maturity and estrous cycle onset is about 4-7 weeks, they are polyestrous and breed year round; ovulation is spontaneous.

1.6.1. Estrous cycle in mice:

The estrous cycle is the time period from the onset of estrous until the onset of the next estrous. The length of the estrous cycle is 4-5 days in mice, but it is highly variable. Identifying the stage of estrous is useful for choosing mice that will mate when paired with a male (to produce timed pregnancy or pseudopregnancy) or tracking the stage of estrous as a variable that may affect research. In the mouse, the estrous cycle is divided into 4 phases (proestrus, estrus, metestrus, and diestrus) and repeats every 4 to 5 days unless interrupted by pregnancy, pseudopregnancy, or anestrus (Allen, 1922). Mouse females may have their first estrous at 25–40 days, and estrous itself lasts about 12 hours, occurring in the evening. Vaginal smears are useful in timed matings to determine the stage of the estrous cycle. Mating is usually nocturnal and may be confirmed by the presence of a copulatory plug in the vagina up to 24 hours post-copulation. The presence of sperm on a vaginal smear is also a reliable indicator of mating.

Estrous cycle is profoundly affected by the day length. In general, breeding mice should be maintained on a 12:12 cycle (12 hours light: 12 hours dark). Interruption of the dark cycle can affect reproductive performance. Ovulation usually occurs 3-5 hours after onset of the dark cycle. Males generally copulate with females in estrous at about the midpoint of the dark period.

Each phase of estrous cycle can be identified by changes in the appearance of vaginal epithelium and vulva. It is possible to identify females in estrous by examining the color,

moistness, and degree of swelling of the vagina. We can summarize the physiological changes in the four phases of estrous cycle as: First, Proestrous, during this period ovarian follicular development occurs. Vagina is gaping and tissues are reddish-pink and moist. Numerous longitudinal folds or striations are visible on the dorsal or ventral lips. Second, Estrous is the time when females are receptive to the male. It lasts for 6-10 hours and ends with ovulation. Vaginal signs are similar to proestrous, but tissues are lighter pink, less moist and striations are more pronounced. Third phase is metestrous, in this period corpus luteum forms and mature eggs moves through oviduct into uterus. Vaginal tissues are pale and dry. Dorsal lip is less edematous. Whitish cellular debri may line the inner walls and/or partially fill the vagina. Forth phase is diestrous which in this period, follicles undergo rapid development for the next ovulation. Ova of previous cycle were eliminated. Vagina has small opening and tissues are blue and very moist.

1.6.2. Gestation period

At fertilization, male spermatocytes penetrate the cellular membrane of the female oocyte (egg). The nuclei of both gametocytes, or sex cells, combine and undergo the process of mitosis. The two cells become one fertilized zygote. Cleavage of the zygote continues and it becomes a morula, a growing mass of undifferentiated cells, after the second day. On the third day, the morula converts to a blastocyst, or a mass of 16 to 40 cells surrounding a blastocoele, which is fluid filled cavity. On day four, the blastocyst implants itself into the uterine lining and becomes an egg cylinder within 24 hours. A placenta forms around the egg cylinder, and it has now become an embryo.

The gestation period in mice varies slightly by strain and ranges from 18-22 days, smaller litters are often carried longer compared to larger litters (Rugh, 1968). Litter size normally ranges from six to twelve. Birth occurs most frequently between the hours of midnight and 4:00 A.M. when animals are maintained under a standard light-dark cycle; however, it can occur anytime of the day or night.

In mice, embryos mature into fetuses after only ten days, the first four of which constitute pre-embryonic development. During embryonic development, the blastocyst

transforms from a mass of cells into an underdeveloped fetus. Fetal development lasts another nine to ten days, and then the mother gives birth to a new born mouse.

1.7 Breeding life expectancy

Young pups reach sexual maturity at the age of two months. Reproductive performance of female mice tends to decrease with increasing age and number of prior pregnancies. Few females of inbred strains, with the exception of FVB/N, will produce more than five litters. Irrespective of past reproductive history, most inbred female's exhibit greatly reduced fecundity by the age of 8-10 months.

Out bred mice and F1-hubrids will routinely surpass inbred strains when comparing reproductive performance. Productive mating (those resulting in live offspring) often approach 100% in out-bred animals. The age of first mating can be as early as 5 weeks and the females may remain fertile up to 18 months of age.

1.8 Role of corpus luteum in gestation

Corpus luteum (yellow body) is a transient endocrine gland that produces essentially progesterone, a required product for the establishment and maintenance of early pregnancy. In the absence of pregnancy, the corpus luteum will cease to produce progesterone, and the structure itself will regress in size over time (Niswender et al., 2000). Corpus luteum consists of two steroidogenic cell types differentiated based on their origin and morphology. Small luteal cells are of the thecal cell origin and respond to LH secretion with increased secretion of progesterone via activation of the protein kinase-A second messenger pathway. Large luteal cells are of granulose cell origin. These cells contain receptors for the PGF2α is mediated through the activation of the protein kinase-C second messenger pathway and luteolytic actions are mediated through process of apoptosis by the activation of the calcium dependent endonucleases (Niswender et al., 2000).

1.9 Role of placenta in pregnancy

The placenta is an organ that connects the developing fetus to the uterine wall to allow nutrient uptake, waste elimination, and gas exchange via the mother's blood supply. The placenta is the first organ to form during mammalian embryogenesis. Problems in its formation and function underlie many aspects of early pregnancy loss and pregnancy complications in humans. Because the placenta is critical for survival, it is very sensitive to genetic disruption, as reflected by the ever-increasing list of targeted mouse mutations that cause placental defects (Rossant and Cross, 2001). The placenta functions as a fetomaternal organ with two components: the fetal placenta, or (Chorion frondosum), which develops from the same blastocyst that form the fetus, and the maternal placenta, or (Decidua basalis), which develops from the maternal uterine tissue. Placenta can be classified into many different types based on the number of layers separating the fetal and maternal blood supply. The categorization was put forth by grosser and later simplified by Steven (Steven and London, 1970). The main placenta types can be characterized as: Epitheliochorial which contains three maternal layers and three fetal layers, for example in pigs, cows, horses and sheep. Endotheliochorial which consists of one maternal layer and three fetal layers, for example found in dogs, seals and ferrets. Hemochorial with no maternal layers and three fetal layers, example for this kind of placenta found in humans, rats and mice.

Placenta based on the macroscopic structure of the sites where attachment occurs between the embryo and endometrium of the uterus is classified into four types. They are Diffuse, as in horse and pig. Cotyledonary, as in ruminants. Zonary, as in carnivorous, cat, and dog. Discoidal placentas, as in human and rodents. Some of the fetal functions that are partially or completely accomplished by placenta during pregnancy are gas transfer, excretory functions, water balance, pH regulation, heat regulation, endocrine, and immunological functions.

The placenta of mice, hamster, rats and guinea pigs produce lactogenic hormones (Greenwald and Rothchild, 1968). A placenta luteotrophic effect can be ascribed to the mouse concepts by the 8^{th} day of gestation, the time at which corpus luteum of pregnancy sharply increase in size.

1.10 Pregnancy and its hormonal control

Hormones are chemical messengers, produced and secreted by organs, which travel via the blood, and exerts some influence upon a target tissue.

The ovaries are organs that are responsible for the development of female gametes. At birth around 400 000 cells have reached prophase of the first meiotic division and are called primary oocytes (often called follicles).

Estrous cycle events are divided into three phases: follicular phase, ovulatory phase, and the luteal phase:

1.10.1 The follicular phase: is the first part of the menstrual cycle, where one or more follicles start to develop into a mature female gamete. The follicle cells surround the oocyte (developing egg cell), and produce hormones that trigger other responses. At the start of the oestrous cycle, the pituitary gland (in the brain) secretes follicle stimulating hormone (FSH) which triggers development of one or more follicles in the ovary, As the follicle grows in size, oestrogen is secreted, it Inhibits further production of FSH, stimulates the pituitary gland to secrete lutenising hormone (LH), and stimulate growth and repair follicular phase of the uterine lining.

1.10.2 The ovulatory phase: is when the oocyte is released (follicle cells remain in the ovary) from the ovary and passes down the fallopian tube and towards the uterus. As the follicular stage progresses, the developing follicle increases in size and becomes a mature follicle, Oestrogen levels increase rapidly, and it triggers further release of LH (high concentration of LH in the blood), and then ovulation will happen, Oocyte leaves the ovary and passes into the fallopian tube, when the female is fertile.

1.10.3 The luteal phase: most of the follicle cells remain in the ovary after ovulation. They continue to develop and form a structure called the corpus luteum, as a result more hormones are produced. The high concentrations of LH that brings about ovulation has an affect on the follicle cells that remain in the ovary, in this phase follicle becomes corpus luteum which secretes some oestrogen and a large amount of progesterone, progesterone stimulates mammary glands and uterus in anticipation of pregnancy, high concentrations of oestrogen and progesterone inhibit production of FSH and LH, without FSH and LH the cells of the corpus luteum gets smaller – and less progesterone and oestrogen is

secreted, with less oestrogen and progesterone, the FSH is no longer inhibited, and the cycle can start again.

In addition to its role in transporting molecules between mother and fetus, the placenta is a major endocrine organ. It turns out that the placenta synthesizes a huge and diverse number of hormones and cytokines that have major influences on ovarian, uterine, mammary and fetal physiology, not to mention other endocrine systems of the mother. Placenta also secrets: Human chorionic gonadotropin, which is a glycoprotein hormone, promotes the maintenance of the corpus luteum during the beginning of pregnancy, causing it to secrete the hormone progesterone. Progesterone enriches the uterus with a thick lining of blood vessels and capillaries so that it can sustain the growing fetus. Due to its highly negative charge, hCG may repel the immune cells of the mother, protecting the fetus during the first trimester. The pituitary analogue of hCG, known as luteinizing hormone (LH), is produced in the pituitary gland of males and females of all ages (Cole, 2009). Placental lactogen is another hormone secretes by placenta and it is a polypeptide hormone. Its structure and function is similar to that of growth hormone. It modifies the metabolic state of the mother during pregnancy to facilitate the energy supply of the fetus.

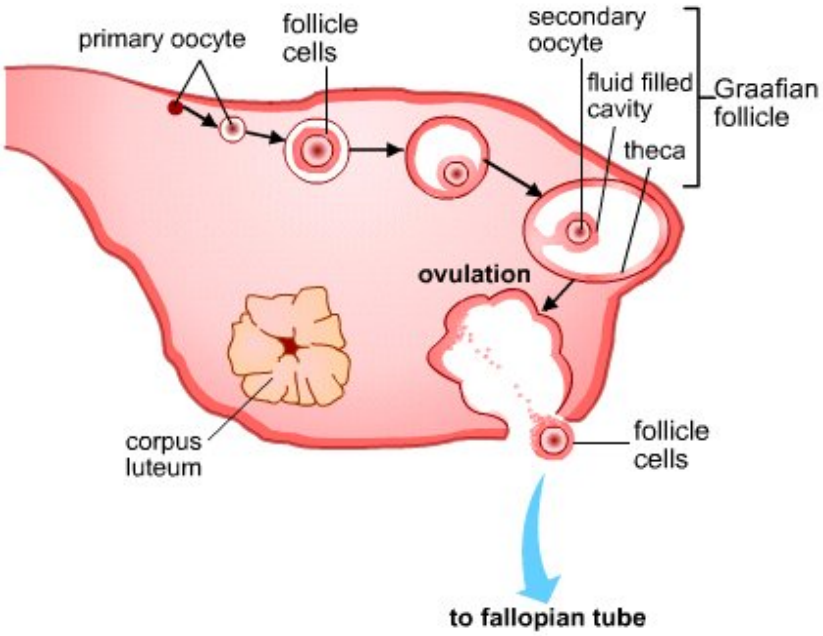


Figure 1.5 Mice ovary during different stages of estrous cycle.

1.11 Hypotheses and research objectives

Myostatin is a critical factor for muscle growth, mouse myostatin-propeptide has been shown to inhibit myostatin activity in vitro and enhance muscle growth in vivo. Myostatin function also appears conserved across different tissues and various vertebrate species, so it is more than just a regulator of muscle mass. Although myostatin has been studied in several ways, its role in vivo unclear especially its role in circulation and during gestation period, studies showed myostatin is expressed in human placenta but its level does not change through different gestational age (Antony et al., 2007), they showed also myostatin is inhibited glucose uptake by placenta tissues. Because only limited data are available in muscles from older mice that exhibit an age-related decrease in muscle mass and function, we hypothesize that: (1) myostatin is expressed in mouse placenta and its level of expression may change through different gestational stages, moreover impact fetal and placental growth; (2) blocking or inhibiting myostatin may prevent muscle loss and fat accumulation in old ages. In order to test these hypotheses, the present project has two research objectives: (1) to investigate myostatin gene expression in mouse placenta and its levels during different stages of gestation and its effects on fetal and placental weight. (2) to investigate effect of myostatin inhibition by its propeptide on muscle loss and fat accumulation in old ages. To achieve objective 1, we used three groups of pregnant mice, wild type group as control, heterozygous transgenic mice ($MSTN^{TG/+}$), and homozygous transgenic mice ($MSTN^{TG/TG}$) in two different stages of gestation, day 10 and day 16. We measured myostatin mRNA expression level by qRT-PCR technique in placenta tissues. To achieve objective 2, we used two groups of mice, wild type as control group and transgenic group, in four different ages, 1 month as growing age, 6 month as young adult, 12 month as middle age, 18 month as aged mice. We also measured the expression of myostatin, MyoD, Myf5, and Pax7 gene.

CHAPTER 2

MYOSTATIN GENE EXPRESSION IN MICE PLACENTA DURING DIFFERENT GESTATION STAGES AND ITS EFFECT ON PLACENTA AND FETAL WEIGHT

2.1 Abstract:

Myostatin, is also called Growth Differentiation Factor 8 (GDF 8), is a member of transforming Growth Factor β (TGF- β) super-family. It is well known as an inhibitor of muscle mass in vertebrates. Over-expression of myostatin in the skeletal muscle of transgenic mice associated with lower muscle mass, myostatin knock-out mice showed significant increase muscle mass. Recent studies have identified myostatin expression in other tissues and species. Myostatin propeptide is a ligand that has been shown to enhance muscle growth by suppressing myostatin function. By transgenic over-expression of myostatin propeptide cDNA transgene, we previously demonstrated significant muscle growth, which is likely the results of increased placental development and fetal growth prenatal. The fetal nutrient supply via the placenta must increase to allow the extra growth of the larger heavily muscled fetus, and this may relate to the metabolic role of myostatin in controlling placental nutrient transport. The aim of the current study was to detect myostatin gene expression in mouse placenta and measuring its level during different gestational stages; moreover, measuring the myostatin influence on placental and fetal weight with some other characteristics. Three groups of mice were used, first group was wild type pregnant female considered as a control group, second, heterozygous transgenic pregnant ($MSTN^{TG/+}$) group, third, homozygous transgenic pregnant ($MSTN^{TG/TG}$) group. Placental tissue samples were taken in day 10 and 16 of gestation. The results showed that transgenic pregnant groups showed significant higher live weight in comparison to their wild type littermates because of carrying significant larger transgenic fetuses and placenta in their uterus, myostatin mRNA showed (21.22±7.31) fold increase in heterozygous and (7.85±1.15) fold increase in homozygous

pregnant groups at day 10 of gestation relatively compared to wild type group, while in day 16 the increased level was (12.28±4.97) and (4.98±0.52) fold in heterozygous and homozygous respectively, a positive correlation was found between myostatin expression level and gestational age in wild type group, while in both transgenic groups this correlation was negative. In conclusion, our present study showed that myostatin express in placenta tissues, and its level changed through different gestational stages, inhibition of myostatin activity increased fetal and placental weight.

2.2 Introduction:

Myostatin is a transforming growth factor-β (TGF-8) superfamily member, known for its ability to inhibit muscle growth. Over expression of myostatin gene in skeletal muscle caused decrease in muscle mass, fiber size, and myonuclear number (Porszasz et al., 2003). The function of myostatin also appears conserved across various vertebrate species because loss-of function mutations in myostatin gene have been found to cause dramatic increase of muscle mass resulting from both muscle hypertrophy and hyperplasia in cattle (Grobet et al., 1997; Kambadur et al., 1997; McPherron and Lee, 1997), human (Schuelke et al., 2004), sheep (Clop et al., 2006) and dog (Mosher et al., 2007). Knock out mice of myostatin gene showed a significant muscle mass increase in comparison to wild-type (McPheron, Lawler, and Lee 1997).

Like other TGF-β family members, myostatin is synthesized as a precursor protein made up of 376 amino acid, consisting of two domains: an N-terminal propeptide and a C-terminal domain considered as the active molecule or the mature domain. Myostatin propeptide, which is generated from the N-terminal propeptide of the myostatin precursor after proteolytic processing, was shown to form a latent complex with mature myostatin both in vitro and in vivo (Lee and McPherron, 2001; Thies et al., 2001; Zimmers et al., 2002). In addition, the mouse myostatin propeptide was demonstrated to enhance muscle growth in vivo by suppressing myostatin function (Lee and McPherron, 2001; Yang et al., 2001). Mature myostatin, following release from its inhibitory proteins, signals via binding to the activin type II receptors (ActRII B) and to a lesser extent with ActRII A (Lee, 2004).

In addition to propeptide, which inhibits myostatin activity, myostatin expression and activity naturally is regulated by a number of proteins include follistatin (Lee and McPheron, 2001), follistatin like-related peptide or FLRP (Hill et al., 2002), growth/differentiation factor-associated serum protein (GASP)-1 (Hill et al, 2003), myostatin-blocking-antibodies (Whittemore et al., 2002) and there is a published paper showed that Titin (T)-cap, which is a sarcomeric protein, binds myostatin with high affinity, and like follistatin, FLRG and GASP-1, can prevent receptor binding and activation (Nicholas et al., 2002).

Myostatin has also shown to have role in glucose homeostasis and adipogenesis (Feldman et al., 2006 and Zhao et al., 2005). Its expression has been detected in different tissues including cardiac (Sundaresan et al. 2008) adipose (McPheron and Lee, 2002) brain (Rodgers and Garikipati 2008) mammary (Manickam et al., 2008) endometrium (Stoikos et al., 2008) uterus (Wong et al., 2009), skin (Zhang et al., 2012) and human placenta (Mitchell et al., 2001).

As the conduit between mother and fetus, the placenta influences the growth and development of the fetus inside uterine through exchange oxygen, hormones, and nutrients diffused through the placental barrier to the fetus. Placental growth affect by maternal health condition, maternal anemia and high body mass index are associated with high placental ratio at birth (placental weight/birth weight) (Godfrey, 2002). Maternal physical activity is important to get new larger offspring size, in sheep farming moving pregnant ewes to different pastures is standard practice, because it leads to the production of larger lambs (Barker et al., 2010). There is a relatively correlation between placenta weight and fetal weight (Risnes et al., 2009). Placental weight correlates with birth weight (Sibley, 1994). In one study placenta weight was increased in parallel to fetal weight (Jansson et al., 2002), whereas placental weight was largely unaffected in the other (Kuruvilla et al., 1994).

Yang et al. 2001 down regulated myostatin gene by myostatin pro-domain over-expression which caused increase in body weight by 17-30% ($P < 0.001$) compared to non-transgenic littermates at 9 weeks of age, this increase in body weight indicates that the fetal nutrient supply via the placenta must increase to allow the extra growth of the larger heavily muscled fetus, and this may relate to the metabolic role of myostatin in

controlling glucose uptake. Therefore, myostatin may control placental size, cellular composition of the placenta, and metabolic transfer of nutrients. In mouse models of obesity and type-2 diabetes, the absence of myostatin protein partially reduced hyperglycemia and adipogenesis indicating that myostatin has a role in glucose metabolism (McPherron and Lee, 2002). Our previous study showed that transgenic mice over-expressing myostatin propeptide are able to maintain normal blood glucose levels and insulin sensitivity under high-fat diet compared with their wild-type littermates, indicating that myostatin may have role in plasma glucose homeostasis (Zhao, Wall, and Yang 2005).

Myostatin can regulate metabolism and glucose uptake in a number of tissues (Antony et al., 2007). It affects glucose uptake independent of insulin and also regulates placental glucose uptake *in vitro* in the human placenta. Maternal under-nutrition during early gestation has been associated with fetal programming for increased risk of metabolic disorders in late life (Mitchell et al., 2006). Hence, the transfer of glucose from the maternal circulation to the fetal is a crucial feature of mammalian development. The placenta plays a key role in this transfer process, which appears to be primarily a function of facilitated glucose transport down a concentration gradient from maternal to fetoplacental compartments (Zhou and Bondy, 1993). We hypothesize that myostatin produce by mice placenta tissue, in an attempt to a better understanding of the physiological importance of myostatin, we designed this experiment to measure the expression level of myostatin mRNA in mice placenta during different stages of gestation and its effect on fetal and placental weight.

2.3 Materials and Methods:

2.3.1 Animals and sample collection:

This study was approved by the institutional animal care and use committee at the University of Hawaii. Myostatin propeptide transgenic mice were generated by standard microinjection techniques, which has been previously described (Yang et al., 2001). All mice were housed in cages with a constant temperature (22°C) and 12-h light/dark cycle,

and were weaned at 4 weeks of age, and given water and food *ad libitum* until 12 weeks, at this age female mice were randomly separated into three groups: first, 14 wild type female (B6SJL) were mated with wild type males to get wild type pregnant group which was considered as a control group. Second, 14 heterozygous transgenic female ($MSTN^{TG/+}$) were mated with wild type males to make heterozygous transgenic ($MSTN^{TG/+}$) pregnant group, third, 14 heterozygous transgenic female were mated with transgenic homozygous males to make homozygous ($MSTN^{TG/TG}$) pregnant group.
Mice were dissected at day 10 and 16 of pregnancy for all three groups, placenta samples were collected and weight, Litter size, fetal weight, uterine length and width were measured. Placentas were put in liquid nitrogen directly after weight, and stored at -80°C for later total RNA extraction.

2.3.2 Genotyping:

Tail samples were collected at age one month, DNA was extracted by phenol/Chloroform extraction method after digesting mouse tail tissue over night 50°C in a buffer containing (10% sodium dodecylsulfate (SDS), 1M Tris PH 8.0, 1M ethylene-diamine tertraacetic acid (EDTA)), and proteinase K (20 mg/ml). The extracted DNA was subjected to polymerase chain reaction (PCR) amplification with a primer set unique to the transgenic mice. The forward and reverse primers were 5`-GACAGCAGTGATGGCTCT-3` and 5`CTTGTCATCGTCGTCCTTGTAATCGGTAC-3`, respectively. PCR conditions were the same as those described previously (Yang et al., 2001). The PCR products were subjected to electrophoresis in a 1% agarose gel and stained with ethidium bromide to examine for the presence of the transgenic PCR product.

2.3.3 Total RNA extraction and cDNA preparation:

Total RNA was isolated from frozen placental samples using TRIzol reagent (Invitrogen, Carlsbad, CA) and chloroform. 100 mg placenta samples were homogenized in TRIzol reagent using a polytron homogenizer at maximum speed for 60 seconds. RNA was re-suspended in diethyl pyrocarbonate-treated (DEPC) water, and the final

concentration of total RNA was determined by measuring absorbance at 260 and 280 nm using a (NanoPhotometer^{TM} P-Class, IMPLEN, P330, Germany). Prior to the reverse transcriptase reaction, total RNA was treated with 1 µl deoxyribonuclease I (Invitrogen) and the appropriate buffers according to protocol to remove any residual genomic DNA. First strand cDNA was synthesized using 5 µg total RNA, 1 µl amplification grade Superscript III Reverse Transcriptase (Invitrogen Life Technologies, Inc.), and the appropriate reaction buffer to a final volume of 20 µl per reaction tube according to the protocol set by Invitrogen. The cDNA was stored at -80°C until use.

2.3.4 Quantitative real-time PCR:

The relative expression of Myostatin was determined by quantitative real-time PCR in an ABI 7300 Sequence Detection System machine (Applied Biosystems, Forrest City, CA). Oligonucleotide primers for myostatin mRNA (Mstn FP, 5`-TGCAAAATTGGCTCAAACAG -3`; Mstn RP, 5`- GCAGTCAAGCCCAAAGTCTC -3`) with amplicon size 182 bp, and β-actin (FP 5`-GACAGGATGCAGAAGGAGATTACT -3`; RP 5`-TGATCCACATCTGCTGGAAGGT -3`) with amplicon size 171 bp. as a reference gene. Each well of the 96-well reaction plate contained a total volume of 20 µl. 2 µl cDNA (1000 fold diluted) solution was combined with 0.5 µl each of forward and reverse primers (10 pmole/µl), 7 µl nuclease free water, and 10 µl SYBR Green PCR Master Mix (Applied Biosystems), all real-time PCR procedures were run in triplicate to correct for variances in loading. All values are expressed as the mean of the triplicate measure for the experimental divided by the mean of the triplicate measure of β-actin for each sample. Optimal annealing temperatures for the primers used was determined to be 60 °C, cDNA with thermal cycle 94°C 4 Minutes and 42 cycles of 94 °C for 40 Seconds, 60 °C for 30 Seconds, and 72 °C for 30 Seconds were run. At the end of the reaction, dissociation curve was created to verify the identity and specificity of the amplification products. All myostatin mRNA values were normalized to an endogenous reference gene (β-actin) and relative to untreated control through the formula $2^{-\Delta\Delta Ct}$.

2.3.5 Statistical Analysis:

Data were analyzed by using a general linear mixed model SAS 9.2 program (SAS Institute, Cary, NC), significance was determined at $P<0.05$, for the differences between the three genotyping group and two different stages of gestation, when a significant interaction was identified, Duncan multiple range program was used for mean comparisons, all data are presented as means ± MSE.

2.4 Results and Discussion:

2.4.1 Detecting of myostatin gene expression in placenta tissue samples:

The aim of the current study was to determine myostatin gene expression in placenta during different stages of gestation and its effect on fetal and placental weight. Gestation period in mice varies slightly by strain and ranges from 19-21 days. In order to determine whether myostatin express in placenta or not, we dissected pregnant female mice in day 10 and day 16 of gestation, placenta samples were saved in freezer (-80°C). The mRNA expression of myostatin and the transgene propeptide in skeletal muscle has been previously detected (Yang and Zhao, 2006) here we show myostatin gene expression in placenta tissues, mRNA extracted from placenta samples, by reverse transcription reaction we could get cDNA, by doing regular PCR we could amplify the myostatin cDNA (182 bp) as shown in figure (2.1). Liver and lung tissues were used as a negative control and muscle tissues as a positive control. β-actin gene expression (171 bp) was showed as a reference gene.

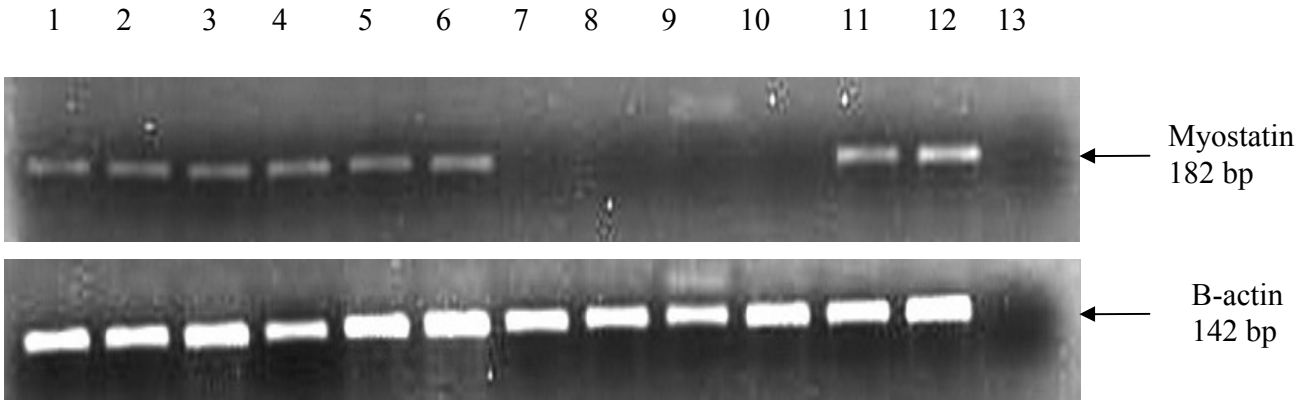


Figure 2.1 Myostatin gene expression and β-actin in placenta tissue, Lane**1**: WT day 10 of pregnancy, Lane**2**: $MSTN^{TG/+}$ day 10, Lane**3**: $MSTN^{TG/TG}$ day 10, Lane**4**: WT day 16, Lane**5**: $MSTN^{TG/+}$ day16, Lane**6**: $MSTN^{TG/TG}$ day 16, Lane**7** & **8**: Liver tissue, Lane**9** & **10**: Lung tissue, Lane**11** & **12**: Skeletal muscle tissue, Lane**13**: no template negative control.

Total RNA concentration showed twice to three times more in placenta tissues (1.44 µg RNA/mg placenta tissue) in comparison to skeletal muscle tissue (0.5 µg RNA/mg muscle tissue) Figure 2.2.

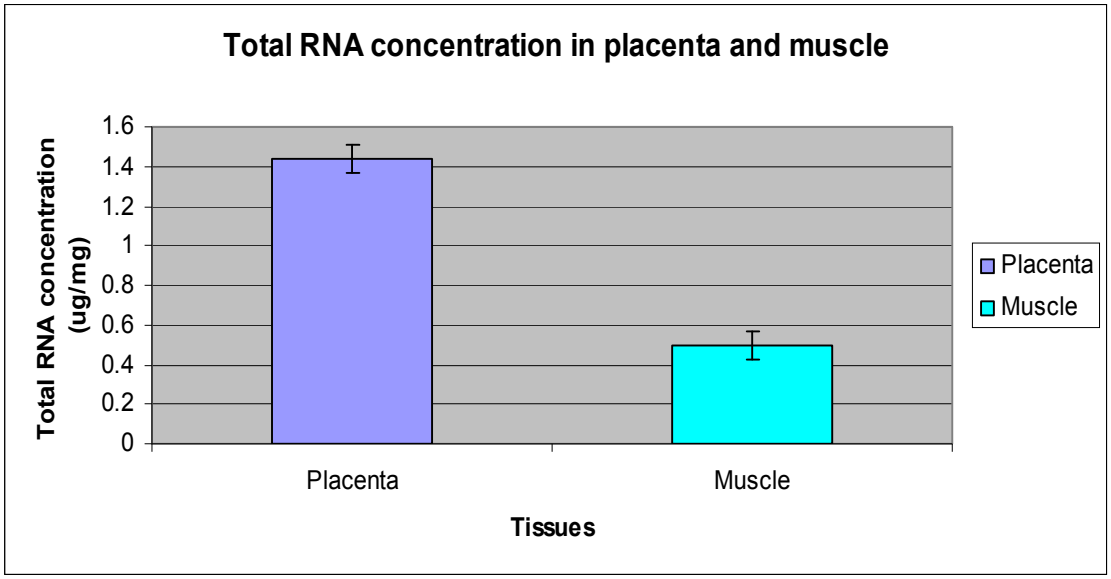


Figure 2.2 Total RNA concentration in placenta and skeletal muscle tissues.

2.4.2 Effect of myostatin on fetal and placental weight during gestation stages and some other characteristics:

Transgenic over-expression propeptide female mice showed 10-20% more muscle mass than wild type (Yang et al., 2001) as it is shown in our data (table 2.1), both transgenic pregnant groups showed significantly ($P<0.05$ and $P<0.01$ for $MSTN^{TG/+}$ and $MSTN^{TG/TG}$ groups, respectively) increase live weight on day 10 of gestation in comparison to WT group and between themselves ($P<0.05$) (table 2.1), this differences were showed continuously on day 16 of gestation between homozygous $MSTN^{TG/TG}$ group and wild type but it was not significant between wild type and heterozygous $MSTN^{TG/+}$ group and within transgenic groups (table2.2). This result indicates that transgenic female groups have heavier fetus in their uterus, which makes their maternal weight heavier.

All pregnant groups did not show any significant differences in the number of fetus or litter size neither for genotype, nor for different gestation stages.

Rates of growth are higher in the neonatal period than during any other stage of postal life, because members of the TGF-β super-family of growth factors play diverse roles in embryonic development as well as in organ homeostasis and injury/pathogen response in adults, so by inhibiting myostatin activity in our experiment, both transgenic $MSTN^{TG/+}$ and $MSTN^{TG/TG}$ total fetal weight were showed significantly, ($P<0.05$), heavier (2.95 ± 0.28, 4.19 ± 0.57 g, respectively) in comparison to WT fetuses (1.22 ± 0.22 g) in day 10 of gestation, but it was not significant within transgenic groups, this difference was continuously appeared in day 16 of gestation in homozygous $MSTN^{TG/TG}$ fetal in comparison to WT and heterozygous group. As total fetal weights were heavier in transgenic fetal Fig 2.3, the individual fetal weights were also heavier in day 10 of gestation for both transgenic groups ($MSTN^{TG/+}$ and $MSTN^{TG/TG}$), it showed significant ($P<0.05$) heavier weight (0.38 ± 0.01, 0.55 ± 0.06 g respectively) in comparison to their WT littermate (0.19 ± 0.04), and significant ($P<0.05$) within tow transgenic groups, this result was in agreement with that obtained by (McPherron et al., 1997) which they revealed that myostatin-null mice produce fetuses larger than controls, these results explain that myostatin may affect fetal growth through fetal nutrition programming, as previous

papers showed that myostatin decrease glucose uptake in human placental BeWo cells (Antony et al., 2007), another study demonstrated that double muscle fetus animals have higher growth hormone receptor gene expression in their muscle tissues compared to normal ones, which they explained the hyperplasia and hypertrophy phenomenon by the concomitantly between growth hormone and myostatin (Listrat et al., 2005). Recently, there are many studies talking about the adverse influences during early development, in particular during fetal life, increase the risk of developing disease in adult life, this paradigm, referred to as fetal programming or developmental origins of health and disease, may have a profound impact on public health strategies for the prevention of major illnesses, there is a strong link between intrauterine environment and adult disease, for example the associations between low birth weight and the risk of developing type 2 diabetes, and cardiovascular disease (Barker, 2006).

Because of the heavy transgenic fetal, uterine weight in maternal pregnant with transgenic fetuses ($MSTN^{TG/+}$ and $MSTN^{TG/TG}$) was significantly ($P<0.05$) heavier (5.88±0.34 and 7.3±0.74 g) respectively, in day 10 of gestation (table 2.1), but not significant within transgenic group, these differences in uterine weight were continuously in day 16 of gestation, homozygous group showed dominant heavier weight in comparison to WT and heterozygous group (table 2.2).

Uterine length affected by genotype, the length increased significantly ($P<0.05$) in transgenic ($MSTN^{TG/+}$ and $MSTN^{TG/TG}$) groups (8.51±0.46 and 9.24±0.68 cm) respectively in comparison to wild type (7.04±0.42 cm), no significant difference between the two transgenic groups, that means because of large transgenic fetal the length of uterine has increased.

Homozygous transgenic ($MSTN^{TG/TG}$) group showed significant increase ($P<0.05$) in uterine width (1.35±0.05 cm) in comparison to WT (1.13±0.07 cm) but there is no significant difference between transgenic $MSTN^{TG/+}$ group (1.26±0.06 cm) with WT and transgenic $MSTN^{TG/TG}$ group (table 2.1), because the transgenic fetuses grew enough during early stages of growth and development, so in day 16 uterine width doesn't show any significant difference, but the individual fetal showed how the transgenic fetuses bigger than WT.

Placental weight is correlated with dietary intake in mammalian pregnancies, so any change in maternal diet will affect the placental-fetal growth and development, maternal under nutrition reduce placental weight and fetal growth. The placenta forms the interface between maternal-fetal circulations and as such is critical for fetal nutrition and oxygenation. In turn, the placenta supply of nutrients to the fetus depends on its size, morphology, blood supply, and transporter abundance. During normal gestation, the placenta goes through many physiological changes, regulated by angiogenic factors, hormones, and nutrient-related genes, to maximize efficiency for an ever-increasing demand for nutrient (Belkacemi et al., 2010). Myostatin showed reduce glucose uptake in placenta, that is indicating that by inhibiting myostatin activity glucose uptake increased, hence placenta and fetus get enough glucose and energy to have this kind of heavy weight in transgenic groups, in our study homozygous transgenic fetuses ($MSTN^{TG/TG}$) have a significant ($P<0.05$) bigger placenta (2±0.21 g) in day 10 of gestation, in comparison to WT (1.1±0.15 g), and significant ($P<0.05$) with heterozygous transgenic $MSTN^{TG/+}$ group (1.5±0.14 g), this heavy placenta result continued in day 16 of gestation which homozygous transgenic group showed significant ($P<0.05$) heavier placenta in comparison to heterozygous and WT groups. Another study reported that placenta weight was increased in parallel to fetal weight (Jansson et al., 2002)

The placenta is responsible to mediate a suitable environment to the fetus for normal growth and development, because adequate placental function is necessary for delivery of nutrients, oxygen, and hormones to the fetus (Risnes et al., 2009). Although placental weight is an important indicator for its function, placental size is correlated with birth size (Salafia et al., 2006), and the ratio between placental weight and birth weight could be a useful indicator for placental efficiency. Placental ratio is the ratio of placental weight to fetus weight, in the present study both transgenic groups ($MSTN^{TG/+}$ and $MSTN^{TG/TG}$) showed significant ($P<0.05$) decrease (0.52±0.06 and 0.55±0.11 respectively) in comparison to WT group (1.02±0.15) without any significant difference within transgenic groups in day 10 of gestation, this low ratio was continued to day 16 in transgenic homozygous group, a comparatively large placenta relative to birth weight may be an expression of a relatively inefficient placenta with reduced ability to translate its own growth into fetal growth. Both animal (McCrabb et al., 1991) and human (Barker

et al., 1993) studies suggest that a placenta that is large relative to birth weight may be a marker for reduced nutrient supply to the fetus. A large US birth cohort study recently showed that a high placenta-to-birth weight ratio, but not birth weight itself, was associated with high blood pressure in childhood (Hemachandra et al., 2006).

Table 2.1 Maternal and fetal measurements on day 10 of gestation for WT, $MSTN^{TG/+}$, and $MSTN^{TG/TG}$ groups.

	Day 10 of gestation		
	WT (n=7)	**$MSTN^{TG/+}$ (n=7)**	**$MSTN^{TG/TG}$ (n=7)**
Live weight (g)	30.61±2.31 a	36.34±1.51 b	42.8±0.59 c
Litter size	7±0.62 a	7.71±0.61 a	7.57±0.37 a
Total fetal weight (g)	1.22±0.22 a	2.95±0.28 b	4.19±0.57 b
Individual fetal weight (g)	0.19±0.04 a	0.38±0.01 b	0.55±0.06 c
Uterine weight (g)	3.48±0.41 a	5.88±0.34 b	7.3±0.74 b
Uterine length (cm)	7.04±0.42 a	8.51±0.46 b	9.24±0.68 b
Uterine width (cm)	1.13±0.07 a	1.26±0.06 ab	1.35±0.05 b
Placental weight (g)	1.1±0.15 a	1.5±0.14 a	2±0.21 b
Placental ratio	1.02±0.15 a	0.52±0.06 b	0.55±0.11 b

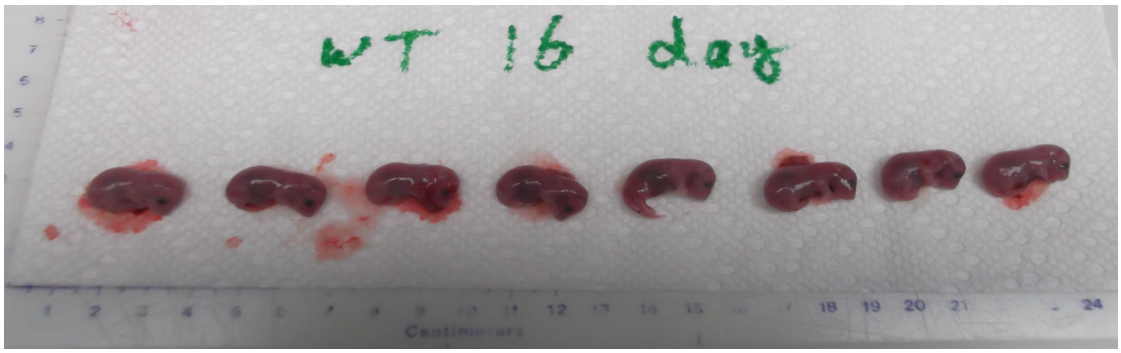
Values are means ± SEM; WT, Wild Type; $MSTN^{TG/+}$, Heterozygous transgenic; $MSTN^{TG/TG}$, Homozygous transgenic; Placenta ratio = total placental weight/ total fetal weight. Means with different letters between different genotype groups significant at ($P<0.05$).

Table 2.2 Maternal and fetal measurements on day 16 of gestation for WT, $MSTN^{TG/+}$, and $MSTN^{TG/TG}$ groups.

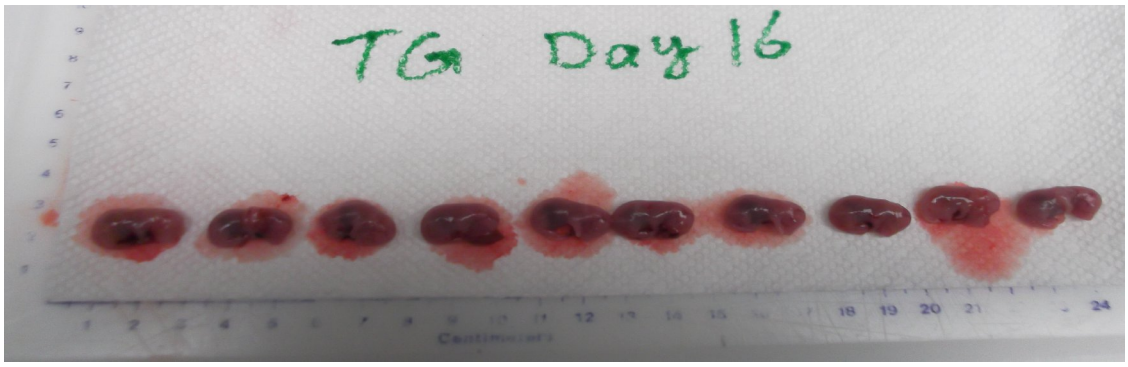
	Day 16 of gestation		
	WT (n=7)	**$MSTN^{TG/+}$ (n=7)**	**$MSTN^{TG/TG}$ (n=7)**
Live weight (g)	39.65±1.62 a	43.13±1.78 ab	46.5±0.24 b
Litter size	8.29±0.52 a	7.71±0.68 a	9.57±0.20 a
Total fetal weight (g)	6.99±0.57 a	8.22±0.26 a	13.53±0.72 b
Individual fetal weight (g)	0.84±0.04 a	1.11±0.09 b	1.41±0.07 c
Uterine weight (g)	10.77±0.69 a	11.39±0.47 a	15.23±0.71 b
Uterine length (cm)	9.46±0.25 a	10.5±0.37 a	13.49±0.58 b
Uterine width (cm)	1.46±0.08 a	1.6±0.08 a	1.6±0.08 a
Placental weight (g)	1.74±0.1 a	1.82±0.17 a	2.43±0.15 b
Placental ratio	0.26±0.03 a	0.22±0.02 a	0.18±0.02 b

Values are means ± SEM; WT, Wild Type; $MSTN^{TG/+}$, Heterozygous transgenic; $MSTN^{TG/TG}$, Homozygous transgenic; Placenta ratio = total placental weight/total fetal weight. Means with different letters between different genotype groups significant at ($P<0.05$).

A



B



C

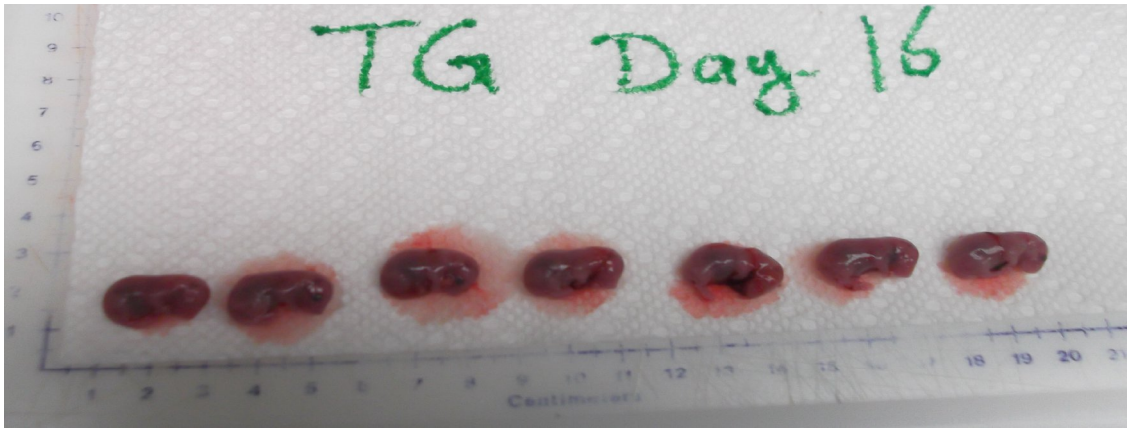


Figure 2.3 Shows phenotype of WT (**A**), MSTN$^{TG/+}$ (**B**), and MSTN$^{TG/TG}$ (**C**) fetuses at day 16 of gestation, TG (homozygous and heterozygous) fetuses showed significant ($P<0.05$) heavier than WT.

2.4.3 Quantitative expression of myostatin on day 10 and 16 of gestation:

Myostatin, a negative regulator of muscle growth, has recently shown to express in other tissues and species, this indicates that myostatin may have physiological roles other than muscle growth. Our results show that mice placental tissues express myostatin throughout gestation and its level increased by (21.22±7.31) fold more in placental tissue of transgenic $MSTN^{TG/+}$ group and (7.85±1.15) fold increase in placental tissue of transgenic $MSTN^{TG/TG}$ homozygous group relatively compared to their wild type WT littermates in day 10 of gestation Fig 2.4.

The qRT-PCR results from the present study of Myostatin mRNA expression in transgenic $MSTN^{TG/+}$ placental tissue showed (12.28±4.97) fold increase and (4.98±0.52) fold increase in transgenic $MSTN^{TG/TG}$ homozygous group in comparison to their wild type (WT) litter mates in day 16 of gestation Fig 2.5. So the increase of myostatin level was continuously in day 16 of gestation in both transgenic groups.

That increase in myostatin level may caused by a negative feedback as blocking myostatin activity in maternal body. Furthermore, the blocking of myostatin activity may cause increase glucose uptake in placenta tissues to supply more nutrient elements or energy for embryo development which cause increase transgenic fetuses weight in comparison to their wild type littermate. Our previous study (Zhao et al. 2005) found that transgenic mice on the high-fat diet showed further enhancement of growth beyond that of the transgenic mice fed a normal-fat diet. Both groups of transgenic mice consumed more kilocalories per day than their wild-type counterparts. Since the majority of the increased weight in transgenic animals was due to increased muscle mass, it indicates that these excess calories were partitioned into muscle mass instead of fat mass, we can come to a conclusion that in the present study the myostatin inhibition by its propeptide caused increased in supplying nutrient elements and energy to the transgenic fetuses to grow more in comparison to their wild type counterparts.

2.4.4 Changes in quantitative expression of myostatin on day 10 and 16 of gestation:

Myostatin mRNA as showed in Fig 2.6, increased by 1.46 fold in day 16 relatively compared to day 10 in WT group. This result was opposite to transgenic groups which showed decreased mRNA expression in $MSTN^{TG/+}$ by -0.99 fold, and -0.71 fold in $MSTN^{TG/TG}$ transgenic group in day 16 in comparison to day 10 of gestation. In another study (Mitchell et al. 2006) they observed no significant differences in the expression of myostatin mRNA in human placental tissues at different gestational stages. The results of the present study may explain the reason of lighter WT fetuses, which is by increasing myostatin levels in WT placenta tissues, glucose uptake decreased in placenta hence the WT fetuses do not get enough nutrient elements and energy so they showed lower weight, but in both transgenic groups as it is shown in the figure 2.6 myostatin level decreased, which may caused increase glucose uptake in placenta, so the transgenic fetuses have enough energy for extra growth and development, depend on the results of other study (Peiris et al., 2010).

2.5 Conclusion:

In conclusion, the results of the current study clearly showed that myostatin express in placenta, its level increased in day 16 of gestation in comparison to day 10 of gestation in WT group, this result was opposite to both heterozygous and homozygous groups which its level decreased in day 16, because previous studies showed that myostatin reduces glucose uptake in placenta, so that is a great support to this idea that transgenic fetuses have enough nutrient elements and energy to show a grater weight in fetuses and placenta.

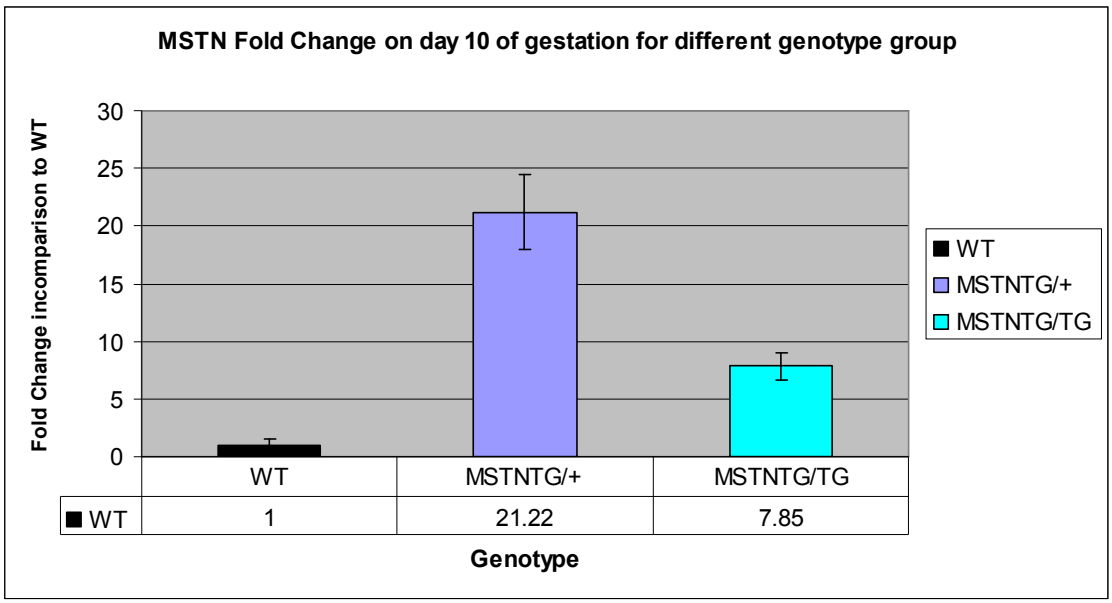


Figure 2.4 Myostatin mRNA expression in placenta, on day 10 of gestation for Wild Type (WT), heterozygous ($MSTN^{TG/+}$), and homozygous ($MSTN^{TG/TG}$) mice groups, were quantified by real time PCR and calculated by $2^{-\Delta\Delta Ct}$. Both transgenic groups showed higher expression (by 21.22 and 7.85 fold in $MSTN^{TG/+}$ and $MSTN^{TG/TG}$, respectively) relatively compared to WT mice.

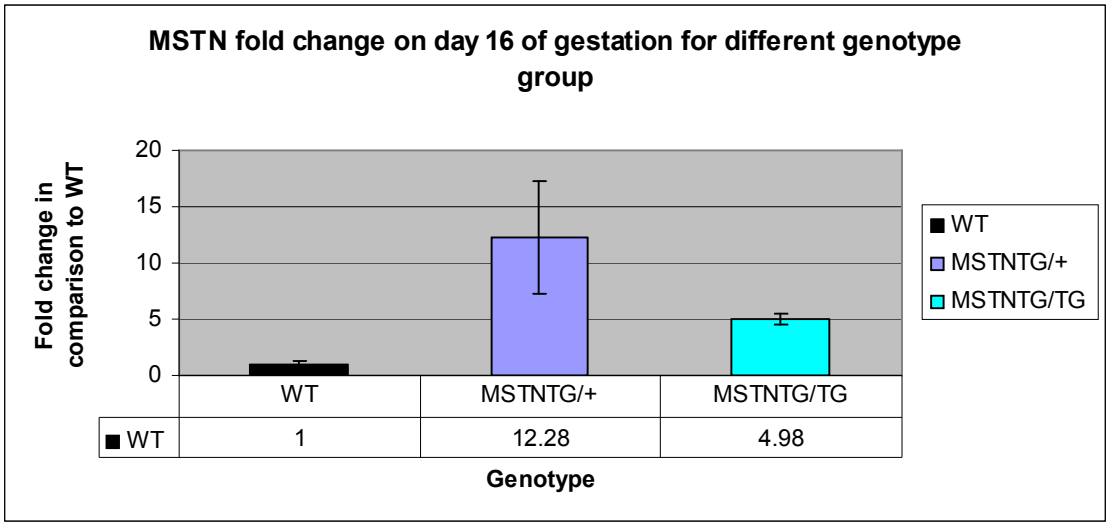


Figure 2.5 Myostatin mRNA expression in placenta, on day 16 of gestation for Wild Type (WT), heterozygous ($MSTN^{TG/+}$), and homozygous ($MSTN^{TG/TG}$) mice groups, were quantified by real time PCR and calculated by $2^{-\Delta\Delta Ct}$. Both transgenic groups showed higher expression (by 12.28 and 4.98 fold in $MSTN^{TG/+}$ and $MSTN^{TG/TG}$, respectively) relatively compared to WT mice.

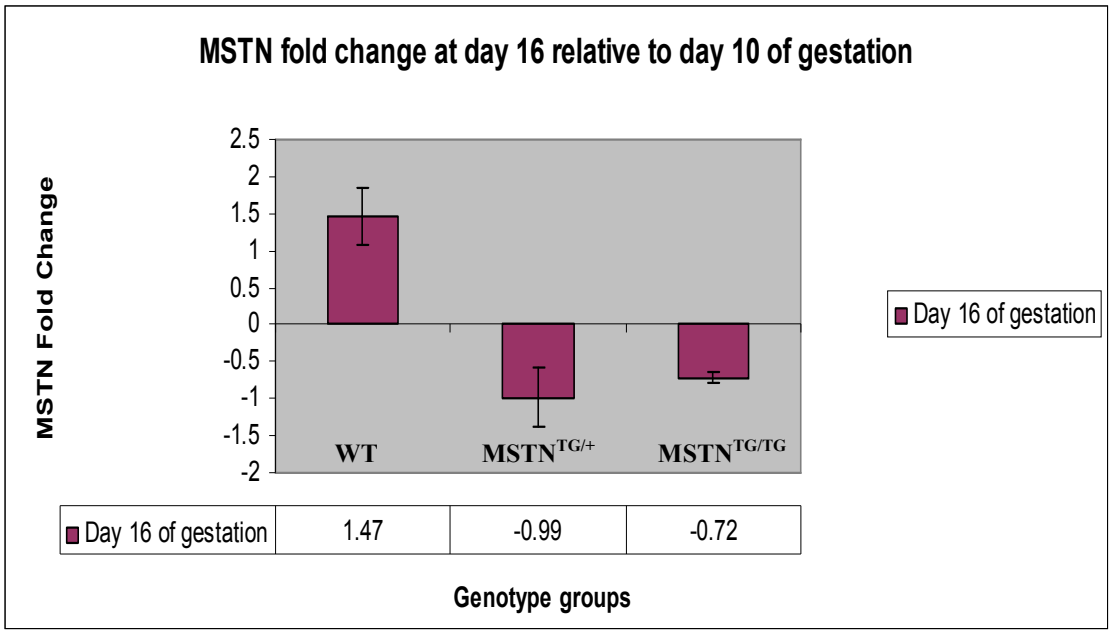


Figure 2.6 Myostatin mRNA expression in placenta, on day 16 of gestation relatively compared to day 10 of gestation for WT, $MSTN^{TG/+}$, and $MSTN^{TG/TG}$ groups were quantified by quantitative RT-PCR and calculated by $2^{-\Delta\Delta Ct}$. WT group showed that myostatin mRNA expressed higher by 1.47 fold in day 16 compared to day 10, while in both transgenic groups, myostatin mRNA level decreased by -0.99 and -0.72 fold in $MSTN^{TG/+}$ and $MSTN^{TG/TG}$ mice respectively, in day 16 compared to day 10 of gestation.

CHAPTER 3

TRANSGENIC EXPRESSION OF MYOSTATIN PROPEPTIDE IN MICE LEADS TO CONTINUE HEAVIER MUSCLE MASS AND PREVENTS FAT ACCUMULATION IN OLD AGES

3.1 Abstract:

A better understanding of skeletal muscle growth is important for agricultural animal production as well as human health. Skeletal muscle responds to body physiological changes such as growth, physical activities and aging by changing its size and composition. Physical activity is considered a significant positive promoter of muscle mass that contributes significantly to the amino acid supplies of the body for immunity and metabolisms. Conversely, loss of skeletal muscle mass through inadequate energy intake, cachexia, and aging has a major impact on human health. Muscle wasting such as sarcopenia is the degenerative loss of skeletal muscle mass and strength associated with aging. One of the transforming growth factors-β superfamily, known as myostatin, is a negative regulator of muscle growth, myostatin knock out mice showed a significant increase in individual muscle mass in comparison to the wild type mice, as well as suppression of body fat accumulation. Myostatin propeptide is a ligand that enhances muscle growth by suppressing myostatin function in transgenic mice. By transgenic over-expression of myostatin propeptide cDNA transgene, we previously demonstrated significant muscle growth. We hypothesized that transgenic mice can continue keeping their muscle mass and suppress adipose tissue in advancing age. We used four different ages of the MLC-pro transgenic and wild type mice: 1month (growing), 6 month (adult), 12 month (middle age), and 18 month (aged mice). The results showed that the transgenic animals have more single muscle weight than the wild-type at old ages. The adipose tissue data showed transgenic mice significantly ($P<0.05$) reduced fat accumulation by 80% compared to wild type littermates in advanced age. qRT-PCR results for gene expression showed myostatin mRNA level increased in transgenic mice and its level

fluctuating between high and low during different time points of age. Each of MyoD and Myf5 mRNA level showed higher fold change in transgenic mice in all time points of our experiment relatively compared to wild-type mice, while their levels reduced gradually with advancing in age. Pax7 gene showed higher expression in transgenic mice compared to wild-type groups at different ages, its level reached the highest expression in 18 months of age in transgenic mice, This finding provides support for the role of myostatin in skeletal muscle maintenance in old animals, and the prospect of targeting myostatin to prevent or reverse progressive muscle wasting that occurs in aging and certain degenerated diseases. Based on the above results myostatin inhibition can enhance and continue skeletal muscle growth in advanced age without accumulation or increase adipose tissue, this dramatic effect of myostatin on post-natal growth is primarily due to the ability of myostatin to negatively regulate skeletal muscle satellite cell activation, proliferation and satellite cell self-renewal through its negative regulation on myogenic regulatory factors and Pax7 gene.

3.2 Introduction:

Skeletal muscle growth and maintenance are essential for human health, as well as have an economic importance for agricultural animals; loss of muscle mass can adversely affect human health and viability, as well as negatively affect the economic benefits in meat production industries as an essential source for animal protein, therefore a better understating of skeletal muscle growth is important by increasing understanding of physiological processes and molecular pathways associated with skeletal muscle growth and development. To date, the majority of the progress made in the field of muscle biology has been accomplished by examining single genes, proteins, or pathways (Reecy et al., 2006). Muscle growth is regulated by a group of growth factors; among them, a member of the transforming growth factor-β (TGF-β) super-family, growth differentiation factor 8 (GDF8), which is also known as myostatin, known for its ability to inhibit muscle growth. Injection of myostatin into mice or over-expression of myostatin in mice significantly reduce muscle mass (Zimmers et al., 2002). Mice with null mutation in myostatin gene causes increase in muscle mass by 20-25% in Belgian

Blue & Piedmontese cattle which is called double muscling phenotype (McPherron and Lee 1997), also in sheep (Clop et al., 2006) in human (Schuelke et al., 2004) and in dog (Mosher et al., 2007).

Myostatin sequence is highly conserved among vertebrate species. Mouse, pig, human, and chicken myostatin are 100% identical in the amino acid sequence of the mature peptide with slight difference in nucleotide sequence (Lee, 2004). Like other TGF-β family members, myostatin is synthesized as a precursor protein, consisting of two domains: an N-terminal propeptide and a C-terminal domain considered as the active molecule or the mature domain. Myostatin propeptide, which is generated from the N-terminal propeptide of the myostatin precursor after proteolytic processing, was shown to form a latent complex with mature myostatin both in vitro and in vivo (Lee and McPherron, 2001; Thies et al., 2001; Zimmers et al., 2002). In addition, the mouse myostatin propeptide was demonstrated to enhance muscle growth in vivo by suppressing myostatin function (Lee and McPherron, 2001; Yang et al., 2001). Mature myostatin, following release from its inhibitory proteins, signals via binding to the activin type II receptors (ActRII B) and to a lesser extent with ActRII A (Lee, 2004).

On the other hand, Knock out mice of myostatin gene showed a significant muscle mass increase in comparison to wild-type (McPheron, Lawler, and Lee 1997) also, in ovo administration of anti-myostatin antibody to chicken, increased post-hatch skeletal muscle growth (Kim et al., 2007). These results led the scientists to think about biological pathway to inhibit that muscle negative regulator, papers showed that myostatin activity can be inhibit by a number of proteins include follistatin (Lee and McPheron, 2001), follistatin like-related peptide or FLRP (Hill et al., 2002), propeptide (Yang et al., 2001), growth/differentiation factor-associated serum protein (GASP)-1 (Hill et al, 2003), myostatin-blocking-antibodies (Whittemore et al., 2002) and there is a published paper showed that Titin (T)-cap, which is a sarcomeric protein, binds myostatin with high affinity, and like follistatin, FLRG and GASP-1, can prevent receptor binding and activation (Nicholas et al., 2002).

Myostatin propeptide is a ligand that enhances muscle growth by suppressing myostatin function in transgenic mice, by transgenic over-expression of myostatin propeptide cDNA transgene. Depend on this information, the inhibition of the production

and activity of myostatin could be a novel potential strategy for the treatment of muscle-wasting disorders, such as muscular dystrophy, cachexia and sarcopenia. The fact that myostatin-inhibited mice have a dramatic increase in muscle mass together with a significant reduction in fat adipose tissue and a depression of adipogenesis (Yang and Zhao, 2006, Zhao et al., 2009), indicating that myostatin may have a role in muscle but also in adipose tissue and this fact could have therapeutic implications in the treatment of obesity.

Because myostatin clearly impacts the formation of muscle, it is necessary to have a basic picture of the process of muscle formation. During the early embryonic stage, muscle progenitor cells from mesoderm-derived somites express myogenic regulatory factors (MRFs), including MyoD, Myf5, myogenin and MRF4, which are a group of transcription factors, expressions of these myogenic transcription factors induce differentiation of muscle stem cells into myoblasts, cells producing a myogenic transcription factor are committed to becoming muscle cells, since transfection of genes encoding any of these myogenic proteins into various cultured cells convert those cells into myoblasts. Myoblasts proliferate until they leave the cell cycle. Then, multiple myoblasts line up together and fuse to form multinucleated myotubes. These myotubes express proteins that allow them to become the contractile units we know as skeletal muscle tissue (Weintraub et al., 1991; Perry and Rudnick, 2000).

In mammals, myofiber numbers are determined before birth; postnatal muscle growth mainly results from muscle hypertrophy. Muscle fiber enlargement could be the consequence of increase in activity of satellite cells, which fuse to adjacent muscle fibers to increase their size. Satellite cells are undifferentiated muscle precursor cells lying between the basal lamina and the muscle fibers, which are responsible for postnatal growth, repair, and maintenance of skeletal muscle (Seale and Rudnicki, 2000). Enlargement of muscle fiber also could result from elevation in muscle protein synthesis, or decrease in muscle protein degradation, or both. IGF-1, which is activated by growth hormone, stimulates both proliferation and differentiation of myoblasts and satellite cells to regulate muscle growth (Oksbjerg et al., 2004). In adults, skeletal muscle regenerative properties decline with age. To test the hypothesis that over-expression of myostatin propeptide prevent muscle loss and fat accumulation in old age, we designed the current

experiment also to measure the expression of some genes related to muscle growth in an attempt to show better physiological understanding of myostatin and some other genes.

3.3 Materials & Methods:

3.3.1 Animals and tissue sampling:

Myostatin propeptide-transgenic mice were generated by standard microinjection techniques, which has been previously described (Yang et al., 2001). Male mice (heterozygous genotype for the transgene) were mated with B6SJL wild type females to produce offspring mice, which were used in this study. Mice were housed in cages; room temperature was maintained at 22°C and 12-h light/dark cycle. Mice were weaned at 4 weeks of age, and given ad-libitum food. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Hawaii. Male mice at 4 different ages: 1 month (growing), 6 month (young adult), 12 month (middle age) , and 18 month (aged mice) with two groups, wild type and transgenic (n=4 to 9 mice per each group), were weighed and sacrificed for muscle tissue dissections and sampling. Gastrocnemius, Soleus, and Plantaris muscle samples immediately dissected out from both legs, cleaned from fat, blood, and weighed. Carcass weight, white adipose tissue (mesenteric fat), brown adipose tissue were measured. All tissue samples cleaned and quickly frozen in liquid nitrogen, and later stored at -80°C freezer until processed.

3.3.2 Genotyping:

Tail samples were collected at age one month, DNA was extracted by phenol/Chloroform extraction method after digesting mouse tail tissue over night 50°C in a buffer containing (10% sodium dodecylsulfate (SDS), 1M Tris PH 8.0, 1M ethylene-diamine tertraacetic acid (EDTA)), and proteinase K (20 mg/ml). The extracted DNA was subjected to polymerase chain reaction (PCR) amplification with a primer set unique to the transgenic mice. The forward and reverse primers were 5`-GACAGCAGTGATGGCTCT-3` and 5`CTTGTCATCGTCGTCCTTGTAATCGGTAC-

3`, respectively. PCR conditions were the same as those described previously (Yang et al., 2001). The PCR products were subjected to electrophoresis in a 1% agarose gel and stained with ethidium bromide to examine for the presence of the transgenic PCR product.

3.3.3 Total RNA extraction and cDNA preparation:

Total RNA was isolated from frozen muscle samples using TRIzol reagent (Invitrogen, Carlsbad, CA) and chloroform. 100 mg gastrocnemius muscle samples were homogenized in TRIzol reagent using a polytron homogenizer at maximum speed for 60 seconds. RNA was re-suspended in diethyl pyrocarbonate-treated (DEPC) water, and the final concentration of total RNA was determined by measuring absorbance at 260 and 280 nm using a (NanoPhotometer^{TM} P-Class, IMPLEN, P330, Germany). Prior to the reverse transcriptase reaction, total RNA was treated with 1 µl deoxy-ribonuclease I (Invitrogen) and the appropriate buffers according to the protocol to remove any residual genomic DNA. First strand cDNA was synthesized using 5 µg total RNA, 1 µl amplification grade Superscript III Reverse Transcriptase (Invitrogen Life Technologies, Inc.), and the appropriate reaction buffer to a final volume of 20 µl per reaction tube according to the protocol set by Invitrogen. The cDNA was stored at -80°C until use.

3.3.4 Quantitative real-time PCR:

Quantifications of mRNA levels for selected genes were performed by quantitative real-time–polymerase chain reaction (qRT-PCR) with SYBR Green reagent in an ABI 7300 Sequence Detection System machine (Applied Biosystems, Forrest City, CA). Primers were designed by primer Express 3.0 software (Applied Biosystems, Foster, CA) and listed in Table 3.1. Each well of the 96-well reaction plate contained a total volume of 20 µl. 2 µl cDNA (1000 fold diluted) solution was combined with 0.5 µl each of forward and reverse primers (10 pmole/µl), 7 µl nuclease free water, and 10 µl SYBR Green PCR Master Mix (Applied Biosystems), all real-time PCR procedures were run in triplicate to correct for variances in loading. All values are expressed as the mean of the triplicate measure for the experimental divided by the mean of the triplicate measure of β-

actin for each sample. Optimal annealing temperatures for the primers used was determined to be 60 °C, cDNA with thermal cycle 94°C 4 Minutes and 42 cycles of 94 °C for 40 Seconds, 60 °C for 30 Seconds, and 72 °C for 30 Seconds were run. At the end of the reaction, dissociation curve was created to verify the identity and specificity of the amplification products. All myostatin mRNA values were normalized to an endogenous reference gene (β-actin) and relative to untreated control through the formula $2^{-\Delta\Delta Ct}$.

3.3.5 Statistical Analysis:

Data were analyzed by using a general linear mixed model SAS 9.2 program (SAS Institute, Cary, NC), significance was determined at $P<0.05$, for the differences between wild type and transgenic groups at each time point of age, and for all weight measurements and gene expression data, when a significant interaction was identified, Duncan multiple range program was used for mean comparisons, all data are presented as means ± SE.

Table 3.1 Primer sequences used for qRT-PCR

Target Gene	Amplicon (bp)	Forward Primer (5-3)	Reverse Primer (5-3)
Myostatin	182	5`- TGCAAAATTGGCTCAAACAG -3`	5`- GCAGTCAAGCCCAAAGTCTC -3`
MyoD	219	5`- GACAGGGAGGAGGGGTAGAG -3`	5`- TGCTGTCTCAAAGGAGCAGA -3`
Myf5	151	5`- AGGAAAAGAAGCCCTGAAGC -3`	5`- GCAAAAAGAACAGGCAGAGG -3`
Pax7	140	5`- GCTACCAGTACAGCCAGTATG -3`	5`- GTAGGCTTGTCCCGTTTCC -3`
β-actin	142	5`- GACAGGATGCAGAAGGAGATTACT -3`	5`- TGATCCACATCTGCTGGAAGGT -3`

3.4 Results:

3.4.1 Effect of myostatin inhibition on muscle growth and adipose tissue at different ages:

In the present study, we measured live weight, single muscle, and adipose tissue weights in different ages for wild type and transgenic mouse. The ages of the mice studied (1, 6, 12, and 18 months) were chosen to represent growing, young adult, middle age and aged mice, respectively. At each time point of age, mice were weighed and sacrificed, muscle tissues were dissected. Muscle weights from transgenic and wild-type littermate mice are summarized in table 3.2. Consistent with our previous report (Yang et al., 2001), transgenic mice showed having significantly ($P<0.05$) more muscle than wild type littermates at almost all time points of different ages. Wild type animals gradually increased their weight and got obesity when getting older (20.4±0.36, 28.97±0.66, 33.71±1.56, and 40.85±3.49 g) in 1, 6, 12, and 18 month of age, respectively. The wild type group increased their weight in 18 month of age by %14 more than transgenic group. This increase may due to that muscle cells die and are replaced by fat cells which swell with aging. In fact it is actually quite possible to be obese and suffering from sarcopenia; while in transgenic animals, muscles keep growing continuously till late of age and there is no fat accumulation in their body at all, the live weights were (22.83±0.58, 35.3±0.77, 37.68±1.39, and 35.16±1.36 g) in 1, 6, 12 and 18 month of age, respectively as shown in table 3.2 and figure 3.1.

The results for the single muscle mass showed that soleus muscle (as a slow twitch muscle) for the transgenic group was heavier in all time points of different ages, especially at age 12 and 18 months reached significant level ($P<0.05$) (12.13±2.51, 9.13±0.32 g, respectively) in comparison to wild type groups at the same ages (9.06±0.45, 7.13±0.38 g, respectively).

The plantaris and gastrocnemius muscle weights (as a fast twitch muscles) for the transgenic group were significantly ($P<0.05$) heavier in all time point of different ages (18±1.54, 32.29±2.01, 30.75±3.28, and 25.83±2 g) and (128.42±3.57, 230.43±7.46, 246.75±5.89, and 175.89±8.97 g) for plantaris and gastrocnemius muscle respectively at

age 1, 6, 12, and 18 months, in comparison to plantaris and gastrocnemius muscles in wild type groups at the same time points of different ages which are (10.78±0.76, 21.07±1.01, 20.19±2.06, and 17.88±0.31 g) and (92.83±3.46, 166.93±7.24, 166.38±13.55, and 151.88±12.72 g) for plantaris and gastrocnemius muscles respectively. As it is shown in table 3.2 Plantaris muscle was heavier by 44% in transgenic mice than in wild type littermates at 18 months of age. Whereas, gastrocnemius muscle was heavier by 16% in transgenic mice than in wild type littermates at old age (18 month).

Transgenic carcass weight showed significant ($P<0.05$) difference at all time point of different ages which was heavier in transgenic mice by 40% at 12 month and heavier by 22% than their wild type littermates at 18 month of age.

White adipose tissue data showed that wild type animals gradually increase their adipose tissue through age, which caused obesity at old age, as it is shown in table 3.2, wild type group significantly ($P<0.05$) increased the their adipose tissue at 18 month of age which is about 80% more than their transgenic littermates, the results of white adipose tissue in transgenic mice interestingly was highly significant as much as when we were dissecting the old transgenic mice there were no white adipose tissue at all.

Brown adipose tissue weight was not significant changed till old age at 18 months which it showed significant ($P<0.05$) increase in wild mice, increased by 22% in comparison to their transgenic littermates as it is showed in table 3.2 and figure 3.7.

3.4.2 Expression of myostatin mRNA, Myogenic Regulatory Factors, and Pax7 through different ages:

The qRT-PCR results showed that myostatin mRNA level fluctuating with advancing in age starting from growing mice (1 month) till the end of our experiment old age mice (18 months), but as general transgenic muscular males have more myostatin mRNA. One month old transgenic animals showed 1.9 fold change (increased), it comes down to 0.95 fold more than wild type animals in 6 months of age, goes up again in 12 month to increase 1.43 fold change, and then finally, comes down to 0.62 fold change in 18 months of age in transgenic animals in comparison to their wild type littermates, figure 3.11.

MyoD mRNA results showed transgenic muscular males have high level of MyoD mRNA in young age compared to wild type mice, and gradually decrease in its levels through age, transgenic mice showed a high level (2.07 fold change) in one month of age, changed to 1.25 fold change in 6 months of age, and then 0.48 fold change in 12 months, finally in 18 months of age the change was 0.43 fold in transgenic mice in comparison to their wild type littermates, figure 3.12.

The results for Myf5 mRNA expression showed higher level in transgenic and younger mice, and gradually decrease in its level with advancing in age, in one month of age transgenic mice showed 1.5 fold increase in the expression level, while in 6 months the expression was 0.5 fold, in 12 months of age the expression level was 0.26 fold decreased, finally at 18 months the difference was 0.007 fold in transgenic mice relatively compared to their wild type littermates figure 3.13.

The gene expression quantification result showed that Pax7 mRNA level was the same in both WT and TG mice at 1 month of age, it increased in 6 months to 1 .66 fold change, it comes back to 1 fold no difference in 12 months, the most interesting result was at the old age (18 month) of their life when the expression level reached 6.12 fold increased in transgenic mice in comparison to their wild type littermates, figure 3.14.

3.5 Discussion:

The aim of the present study was to measure the effect of myostatin inhibition on muscle wasting with advancing age, in an attempt to better understanding physiologically how myostatin mRNA level change through different ages and measuring how inhibition of myostatin by its propeptide prevents muscle wasting and fat accumulation in old age, we measured myostatin mRNA levels, with some other genes related to muscle proliferation and differentiation during four different ages for wild type and transgenic mouse groups. We used four different ages of mice (1, 6, 12, and 18 months) to represent growing, young adult, middle age, and aged mice, respectively.

The transforming growth factor-β (TGF- β) super-family encompasses a large group of growth and differentiation factors playing important roles in regulating embryonic development and in maintaining tissue homeostasis in adult animals. Myostatin (TGF-8), as a member of TGF- β, is a negative regulator of skeletal muscle growth, in our previous report (Yang et al., 2001) we showed that over-expression of myostatin propeptide cDNA dramatically enhanced skeletal muscle development, most likely by maintaining myostatin in its latent form; the increased muscle mass in the transgenic muscle mass most likely due to hypertrophy, or increased in muscle cross-sectional size, rather than hyperplasia. Also noted is that the transgenic mice demonstrated much lower epididymal fat pad weights than their wild type littermates.

Here for the first time to our animal model of transgenic myostatin inhibition, we showed the skeletal muscle growth and maintenance in old age, with no fat accumulation. The live weight measurements clearly showed that wild type animals gradually and significantly ($P<0.05$) increased their weight with advancing age which was increased by 14% more than transgenic littermates in 18 months of age, this increase is due to fat accumulation and the muscle cells die and are replaced by fat cells which swell with aging. In fact it is actually quite possible to be obese and suffering from sarcopenia; which is the progressive loss of skeletal muscle mass and function with advancing age, leading to reduced mobility and quality of life. Sarcopenia is also associated with an increased incidence of metabolic disorders, including obesity and diabetes (Roubenoff et al., 1998).

At age of 18 months mice showed a significant ($P<0.05$) loss of muscle mass and function compared with younger mice, table 3.2. The reduction in muscle mass is associated with fiber atrophy, a loss of muscle function, and an increased susceptibility to contraction-mediated damage (Faulkner, Brooks, and Zerba, 1995). This reduction in muscle mass was clearer in wild type mice more than transgenic groups because they already have less muscle mass so relatively when they loss even less they will become less viable and look obese, for example Soleus muscle (as slow twitch muscle) showed significant ($P<0.05$) reduction by 21% and 24% from age 12 month to 18 months in wild type and transgenic group respectively. Soleus muscle reached the peak at age 12 months in both groups, the transgenic mice have significantly ($P<0.05$) heavier Soleus muscle at middle and old age, table 3.2 and figure 3.2.

Plantaris muscle (as fast twitch muscle) was significantly ($P<0.05$) affected by different age and genotype, reached the peak at 6 months of age in both groups, transgenic mice have significant ($P<0.05$) heavier plantaris muscle by 52% and 44% at middle and old age respectively, plantaris as a fast twitch muscle starts dying first in advancing in age more faster than slow twitch muscles, that is why it starts losing early after it reaches the peak at 6 months of age, it lost 13% and 16% of its mass from 12 months to 18 months in wild and transgenic mice respectively as shown in table 3.2 and figure 3.3.

Gastrocnemius muscle is another example of fast twitch muscle showed significant difference ($P<0.05$) between wild type and transgenic mice at all time points of our experiment. Gastrocnemius muscle reached its peak in middle age, the transgenic mice showed significantly ($P<0.05$) heavier by 48% and 16% at middle and old age respectively, compared to their wild type littermates, the reason of heavier muscle may due to myostatin inhibition which completely prevented the age-related decrease in relative body mass, increased the mass of selected muscles, later starts reduction gradually by 9% and 29% in wild type and transgenic mice from middle to old age.

The transgenic mice showed heavier carcass weight by 40% and 22% at middle and old age respectively compared to wild type; it reached the peak at middle age (12 months) in both groups, and then started losing its mass by 8% and 20% in wild type and

transgenic mice from middle age to old age respectively, table 3.2, figure 3.5, and figure 3.8.

These results clearly provide evidence that myostatin inhibition supported continues muscle build-up in old age skeletal muscle tissue, this result was in agreement with which we obtained in our previous report (Zhao et al., 2009) that the main muscles from transgenic mice weighed significantly more than those from wild type littermates at one year old mice, we observed that the percentage increase in main muscles of transgenic mice over the wild-type mice ranged from 76% to 152%, for further understanding of the reason of this phenomenon the result of staining the muscle fiber showed increased fiber size and muscle fusion in transgenic mice, moreover, the detailed observations of the myofiber histology indicated more nuclei were localized in the central and basal lamina of the myofibers of the transgenic mice, another reason of losing muscle mass in old age may due to increase catabolic reaction in the body, on the other hand, in advancing age the appetite and amount of food intake automatically decrease, so the energy and nutrient intake decrease, so protein degradation level increase these are happening more in wild type mice.

The interactions that occur between skeletal muscle and adipose tissue play a significant role in growth and development which include utilization of energy substrates, muscle growth, and energy storage. As the main location for storage of energy for metabolism, adipose tissue releases energy at necessary times for use by muscle and other parts of the body. Since muscle and adipose tissue develop from the same mesenchymal stem cells, we hypothesized that myostatin gene inhibition may cause a switch between myogenesis and adipogenesis. The myostatin gene is expressed at low levels in adipose tissue and myostatin protein is found in circulation suggesting that myostatin could have a direct role in regulating adipocyte differentiation or function (Lee, 2004). The results of the current study showed a very high significant difference ($P<0.05$) between wild type and transgenic mice at 18 months of age, it reached a level that during dissecting you can directly decide which one is transgenic and which one is wild type without doing any genotyping procedure it is clearly showed in figure 3.10, because the amount of adipose tissue accumulated at their abdomen (mesenteric fat) in wild type mice which was more by 80%, the white adipose tissue weight in old age (18 months) was 1129.5±87.56 g,

while in their transgenic littermates at the same age was 215.67±43.81 g, it was not significant at other time points. That means transgenic mice have 80% less fat figure 3.10. This result supports our hypothesis that myostatin inhibition affects adipose tissue mass in addition to skeletal muscle mass. Specifically, myostatin-inhibited mice have a significant reduction in fat accumulation with increasing age, table 3.2 and figure 3.6.

The result for brown adipose tissue showed the same change, it was significantly reduced in transgenic mice to 139.72±13.66 g compared to their wild type littermates 179.75±16.28 g at 18 month of age; it was reduced by 22%. This result was in agreement with that obtained by (McPherron and Lee 2002) they showed that loss of myostatin function prevents age-related adipose tissue accumulation and positively affects serum glucose and insulin levels. There are two theories may explain how myostatin inhibition affect fat deposition and accumulation, first, is that myostatin has a direct effect on adipose tissue. The second is indirect effect which by increasing in skeletal muscle mass utilizing more fuel and increase metabolic rate, hence there is less energy available to be stored as fat. The other explanation for indirect effect of myostatin is that the lack of myostatin in muscle affects the activity of a hypothetical second messenger released by muscle that acts on adipose tissue (McPherron and Lee, 2002). The reduction of fat accumulation in myostatin propeptide transgenic mice raised the possibility that inhibition of myostatin might be an effective method of suppressing the development of obesity in settings of abnormal fat accumulation.

Myostatin mRNA expression level increases under some atrophy-inducing conditions, such as hindlimb unloading (Carlson et al., 1999; Wehling et al., 2000), HIV-infection (Gonzalez-Cadavid et al., 1998), microgravity environment (Lanai et al., 2000), and cachexia (Zimmers et al., 2002). Age related skeletal muscle wasting has relation with other side effects such as falls, fractures, and death and therefore has important socioeconomic consequences. The molecular mechanisms controlling age-related muscle loss in humans are not well understood, but are likely to involve multiple signaling pathways. It has been found that both myostatin mRNA and protein levels are significantly elevated in old rats, suggesting an involvement of the protein in age related muscle wasting (Leger et al., 2008). In the present study, qRT-PCR results showed myostatin mRNA level changed in transgenic mice by 1.9, 0.94, 1.44, and 0.08 fold at 1,

6, 12, and 18 months of age compared to their wild type littermates, as it is shown in figure 3.11. Its levels are fluctuating between high and low during different time points of age. The myostatin mRNA level was increased by 12 fold in wild type mice at 18 months when compared to 1 month of age while in transgenic mice was 3.47 fold change (figure 3.12), so that means in advancing age myostatin mRNA level increases, this result was in agreement with that obtained by (Lania et al., 2000) which they observed that myostatin mRNA and protein increased by 1.9 to 5 fold in 17 days of space flight that produced 19-24% atrophy of muscles of the lower hind leg in rats, consistent with myostatin being a negative regulator of muscle mass. (Wehling et al., 2000) found increase in myostatin mRNA and protein by 110% and 37% respectively in rat, when hind leg was unloaded for 10 days which produced 16% atrophy of the plantaris muscle. When a 30 minute period of muscle loading was superimposed during the unloading period, the loss of muscle mass was totally prevented although the increase in myostatin expression was only blunted by 50%. It was concluded that although increases in myostatin accompany muscle atrophy, significant increases in myostatin do not necessarily produce muscle atrophy (Wheling et al., 2000). The reason of producing more myostatin mRNA in transgenic mice may due to a negative feedback of muscle fibers when they could not get enough amount of myostatin protein because of inhibition by its propeptide.

Myogenic regulatory factors (MRFs) including MyoD, Myf5, myogenin and MRF4, are a group of transcription factors that contain a conserved E-box DNA- binding domain. During myogenesis, MyoD and Myf5 are redundant in myoblast specification whereas myogenin with either MyoD or Mrf4 are required for differentiation (Buckingham, 2006). Myogenin is associated with terminal differentiation and fusion of myogenic precursor cells to new or existing fibers. When satellite cells are activated, cell-cycle markers, MyoD and Myf5 transcripts are detectable. Subsequent satellite cell differentiation is marked by the appearance of myogenin (Buckingham, 2006). Myostatin known for its role in inhibiting MyoD expression and activity via Smad3, which blocks myoblasts from differentiating into myotubes (Langley et al., 2002) Therefore, myostatin inhibits both myoblast cell proliferation and differentiation. Because MyoD is a key regulator of genes involved in the initiation and maintenance of differentiation in muscle, it is likely that myostatin, through MyoD, inhibits the muscle gene expression program. As it is shown in

our present results by inhibiting myostatin, MyoD mRNA changed by 2.07, 1.25, 0.48, and 0.43 fold at 1, 6, 12, and 18 months of age respectively in transgenic compared to wild type mice, the expression reduced gradually with advancing age, figure 3.12. These results demonstrate that in addition to proliferation, myostatin also negatively regulates myoblast differentiation; myostatin signaling specifically induces Smad 3 phosphorylation and increases the interaction of Smad 3 with MyoD. Furthermore, dominant-negative Smad 3 interferes with the myostatin down-regulation of the MyoD promoter. This result was in agreement with that obtained by (Langley et al., 2002) which they revealed that after 4 days of incubation in differentiation media, control myoblasts, without myostatin treatment, showed a significant increase in MyoD expression compared with myoblasts in growth media. By contrast, MyoD expression was not increased in the myostatin-treated C2C12 myoblasts cultured in differentiation media, they reported that MyoD can be down-regulated by myostatin even after it has been induced and the myogenic differentiation program has been initiated. In wild type mice the MyoD gene expression showed decreasing associated with advancing in age compared to 1 month age as growing mice figure 3.14.

Myf5 mRNA expression was also examined through different time point of mice age, the qRT-PCR result showed that Myf5, like MyoD was up-regulated by inhibiting of myostatin, which its level was higher in transgenic mice, and younger age but with advancing in age gradually its level reduced, its level in transgenic mice was changed by 1.5, 0.5, 0.26, and 0.007 fold at 1, 6, 12, and 18 months of age, respectively, compared to wild type littermates, figure 3.13. The same results obtained by (Langley et al., 2002) when they observed that the addition of myostatin at all time points after the switch to differentiation media down-regulated both Myf5 and myogenin compared with controls suggesting that, like MyoD, Myf5 and myogenin expression can be inhibited even after induction. These results suggest that myostatin inhibits both the up-regulation and expression of MRFs and MHC in myoblasts cultured under differentiating conditions. Wild type mice showed reduce Myf5 gene expression associated with old age when compared to growing young (1 month) age figure 3.16.

Repair and maintenance of skeletal muscle is attributed to the skeletal muscle stem cell pool, the satellite cells. Satellite cells represent a unique population of muscle

precursor cells that are located between the basal lamina and sarcolemma of adult myofibers (Bischoff and Heintz, 1994, and Grounds and Yablonka-Reuveni, 1993). In response to several stimuli including muscle injury, quiescent satellite cells activate, proliferate and differentiate to repair damaged skeletal muscle (Bischoff, 1989). MyoD and Myf5 as myogenic regulatory factors are critical for myogenic specification. However, recent evidence has implicated the paired box transcription factor Pax7 in specification of the myogenic lineage. MyoD, Myf5, and Pax7 appear to play critical roles during satellite cell activation, proliferation, and differentiation. In fact Myf5 expression is present in the majority of quiescent satellite cells (Beauchamp et al., 2000). Similarly, Pax7 is expressed in quiescent satellite cells and moreover, is transcriptionally active in these quiescent cells (Seale et al., 2000 and Zammit et al., 2006). Following activation, satellite cells co-express Pax7 and MyoD (Seale et al.,2000b, Asakura et al., 2001, and Yablonka-Reuveni and Rivera, 1994), proliferate and then down-regulate Pax7 prior to differentiation (Olguin and Olwin, 2004 and Zammit et al., 2004). In our study transgenic mice showed higher expression of Pax7 mRNA by 1, 1.66, 1.07, and 6.12 fold at 1, 6, 12, and 18 months of age respectively, relatively compared to their wild-type littermates, figure 3.11, as the qRT-PCR result showed that transgenic old mice showed very high fold change (6.12) of Pax7 gene which might be the reason of activating, proliferation and differentiation of quiescent satellite cells and fusion to myofibers in transgenic mice. Increasing the expression of Pax7 at old age (18 month) coincided with decreasing of MyoD gene, as it has been mentioned by (McFarlane et al., 2007) that Pax7 inhibit MyoD gene expression. Wild type mice showed decrease Pax7 gene expression with advancing in age when compared its level with 1 month of age as young growing mice as it is showed in figure 3.18.

3.6 Conclusions:

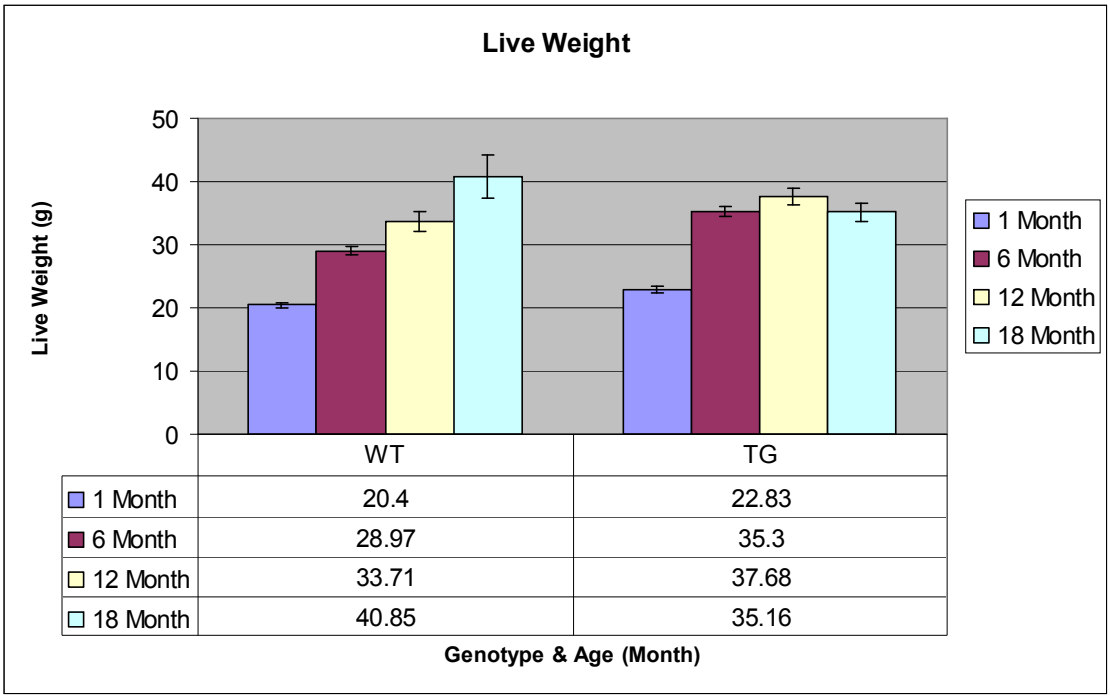
The results of the present study showed that myostatin inhibition has a dramatic increase in muscle mass together with continued muscle growth in the old age which may have therapeutic implications in the treatment of sarcopenia, on the other hand, myostatin inhibition has a significant reduction in fat depots and a depression of adipogenesis,

points towards myostatin having a role not only in muscle but also in adipose tissue and this fact could have therapeutic implications in the treatment of obesity.

Based on the observations on gene expression, we believe that myostatin negatively regulate skeletal muscle proliferation and differentiation, and our results clarify that the dramatic effect of myostatin on post-natal growth is primarily due to the ability of myostatin to negatively regulate skeletal muscle satellite cell activation, proliferation and satellite cell self-renewal through its negative regulation on myogenic regulatory factors and Pax7 gene.

Table 3.2 Whole body weights, muscle wet weight, carcass, and adipose tissue weight in transgenic and wild type mice of different age (mean ± SEM). Means with different letters between WT and TG significant at ($P<0.05$).

	1 month old		6 month old		12 month old		18 month old	
	WT (n=9)	TG (n=6)	WT (n=7)	TG (n=7)	WT (n=8)	TG (n=4)	WT (n=4)	TG (n=9)
Live Wt. (g)	20.4±0.36 a	22.83±0.58 a	28.97±0.66 a	35.3±0.77 b	33.71±1.56 a	37.68±1.39 a	40.85±3.49 a	35.16±1.36 b
Soleus (mg)	4.39±0.27 a	6.17±0.4 a	7.36±1.04 a	8.64±0.7 a	9.06±0.45 a	12.13±0.51 b	7.13±0.38 a	9.13±0.32 b
Plantaris (mg)	10.78±0.76 a	18±1.54 b	21.07±1.01 a	32.29±2.01 b	20.19±2.06 a	30.75±3.28 b	17.88±0.31 a	25.83±2 b
Gastro (mg)	92.83±3.46 a	128.42±3.57 b	166.93±7.24 a	230.43±7.46 b	166.38±13.55 a	246.75±5.89 b	151.88±12.72 a	175.89±8.97 b
Carcass wt. (g)	6.87±0.12 a	8.95±0.33 b	12.26±0.34 a	17.14±0.47 b	13.03±0.78 a	18.27±0.37 b	11.93±0.82 a	14.55±1.07 b
White fat (mg)	152.22±16.18 a	173.33±9.13 a	326.86±31.51 a	181.57±29.48 a	808.88±127.21 a	682.5±36.77 a	1129.5±87.56 a	215.67±43.81 b
Brown fat (mg)	88.11±4.93 a	112.67±17.48 a	116.29±7 a	124.67±8.31 a	167±15.79 a	164.75±14.29 a	179.75±16.28 a	139.72±13.66 b



	WT	TG
1 Month	20.4	22.83
6 Month	28.97	35.3
12 Month	33.71	37.68
18 Month	40.85	35.16

Figure 3.1 Live weights (g) of wild type and transgenic male mice at four different ages, showing gradually weight increase in wild type mice with advancing in age because of fat accumulation in their body and getting obesity, while transgenic group continue muscle growth without fat accumulation.

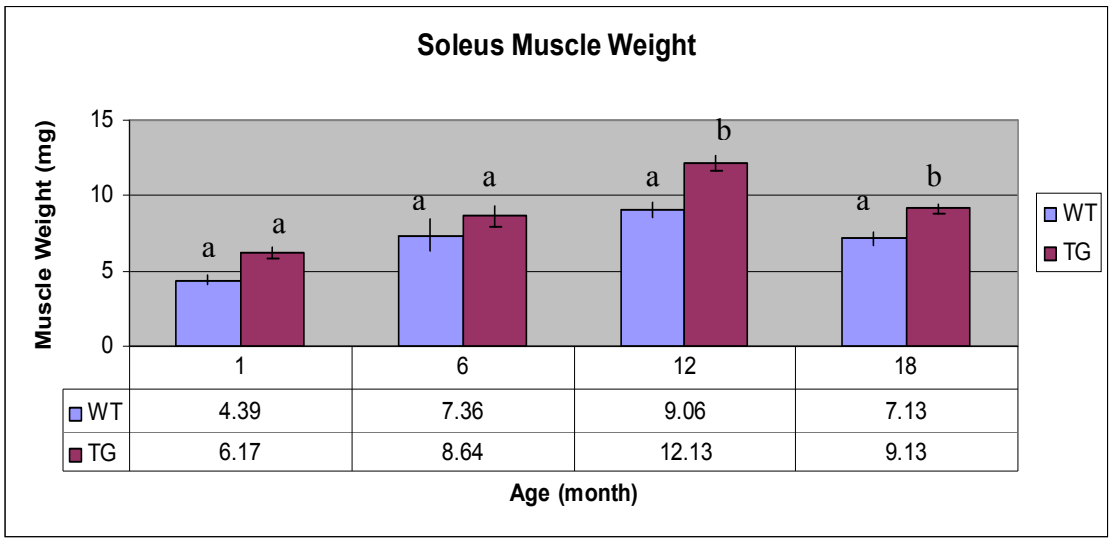


Figure 3.2 Soleus muscle weight (mg) (Slow twitched muscle) in wild type and transgenic male mice at four different ages, showing higher muscle weight in transgenic groups at all time points compared to their wild-type littermates. Means with different letters between WT and TG significant at ($P<0.05$).

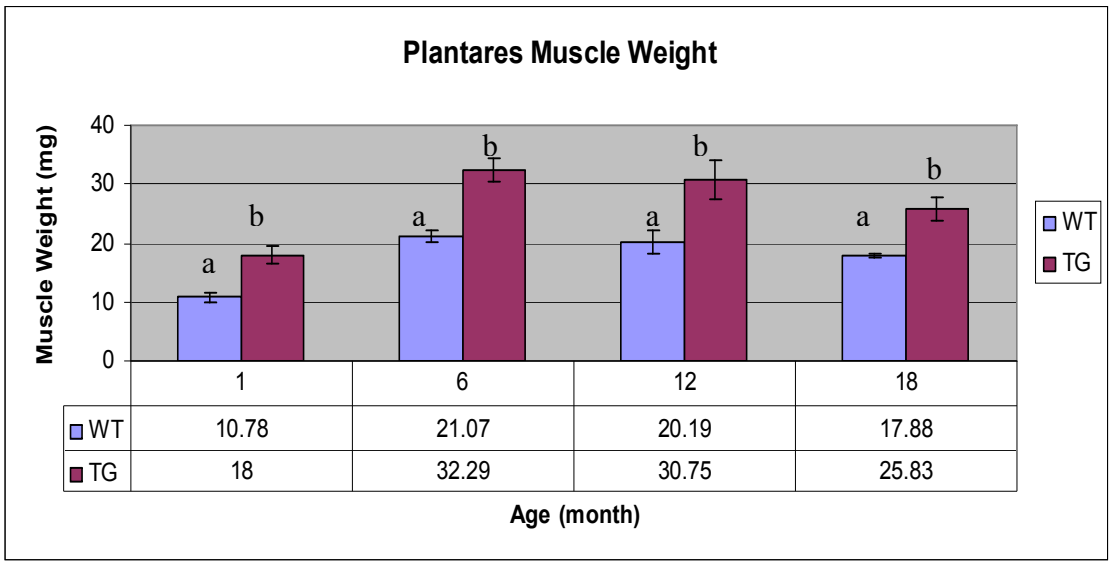


Figure 3.3 Plantaris muscle weight (mg) (Fast twitched muscle) in wild type and transgenic male mice at four different ages, showing higher muscle weight in transgenic groups significantly at all time points compared to their wild-type littermates. Means with different letters between WT and TG significant at ($P<0.05$).

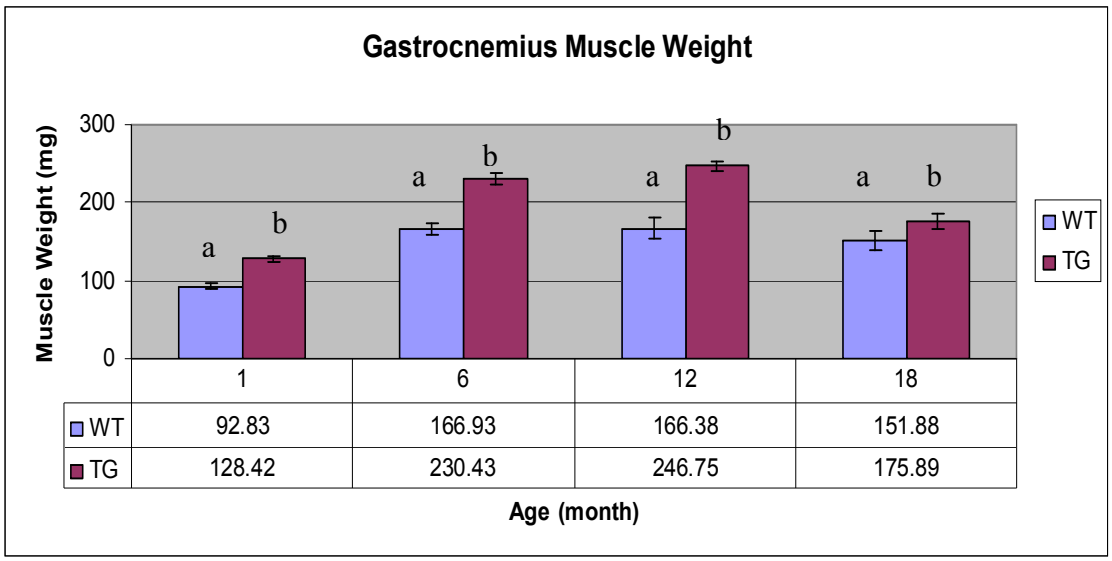


Figure 3.4 Gastrocnemius muscle weight (mg) (Fast twitched muscle) in wild type and transgenic male mice at four different ages, showing higher muscle weight in transgenic groups at all time points compared to their wild-type littermates. Means with different letters between WT and TG significant at ($P<0.05$).

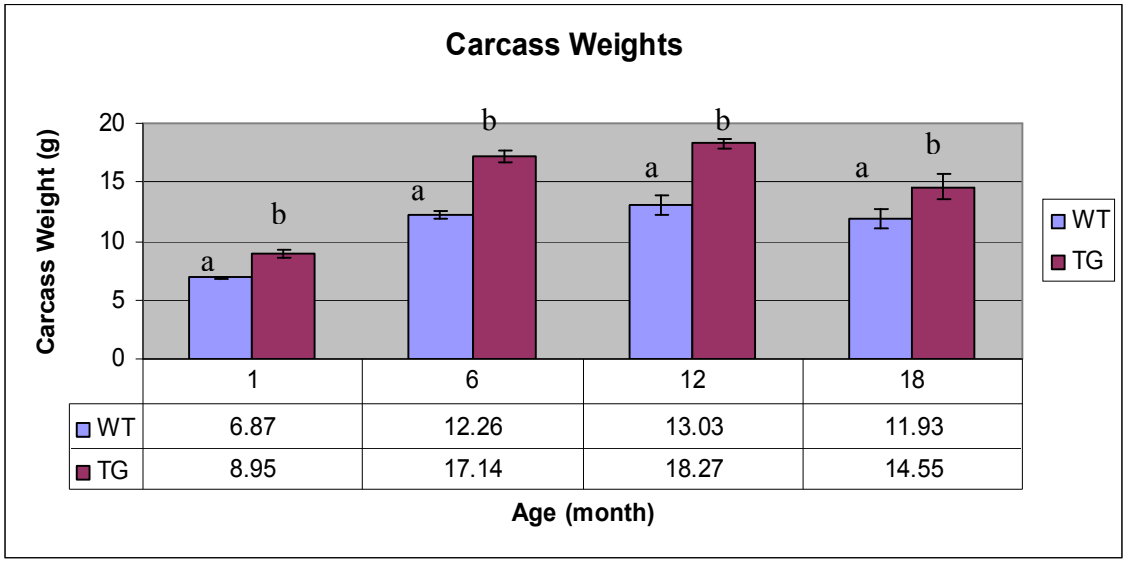


Figure 3.5 Carcass weight (g) in wild type and transgenic male mice at four different ages, showing higher muscle weight in transgenic groups at all time points compared to their wild-type littermates. Means with different letters between WT and TG significant at ($P<0.05$).

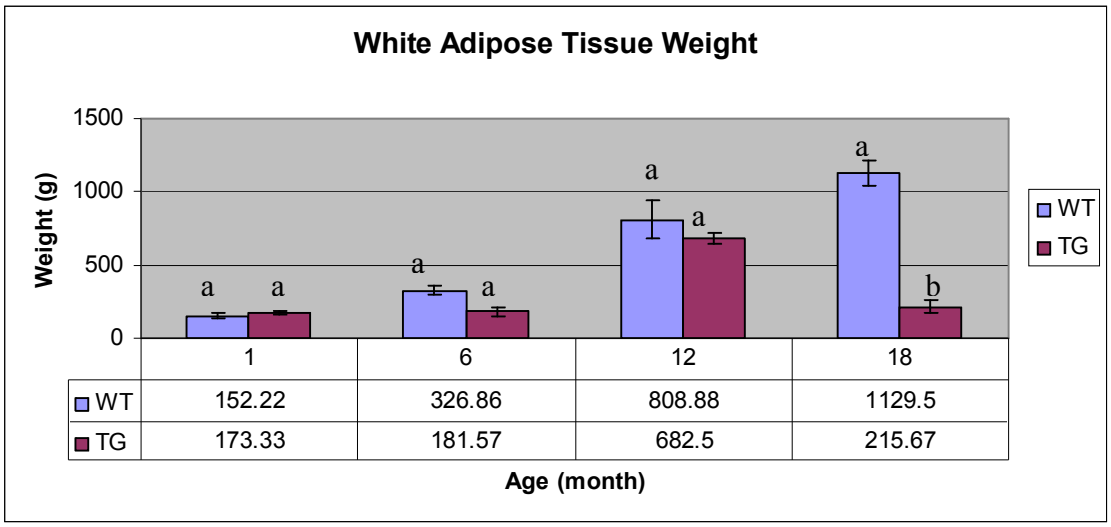


Figure 3.6 White adipose tissue weight (g) in wild type and transgenic male mice at four different ages, showing significant reduced weight in transgenic type group at 18 months of age compared to their wild-type littermates. Means with different letters between WT and TG significant at ($P<0.05$).

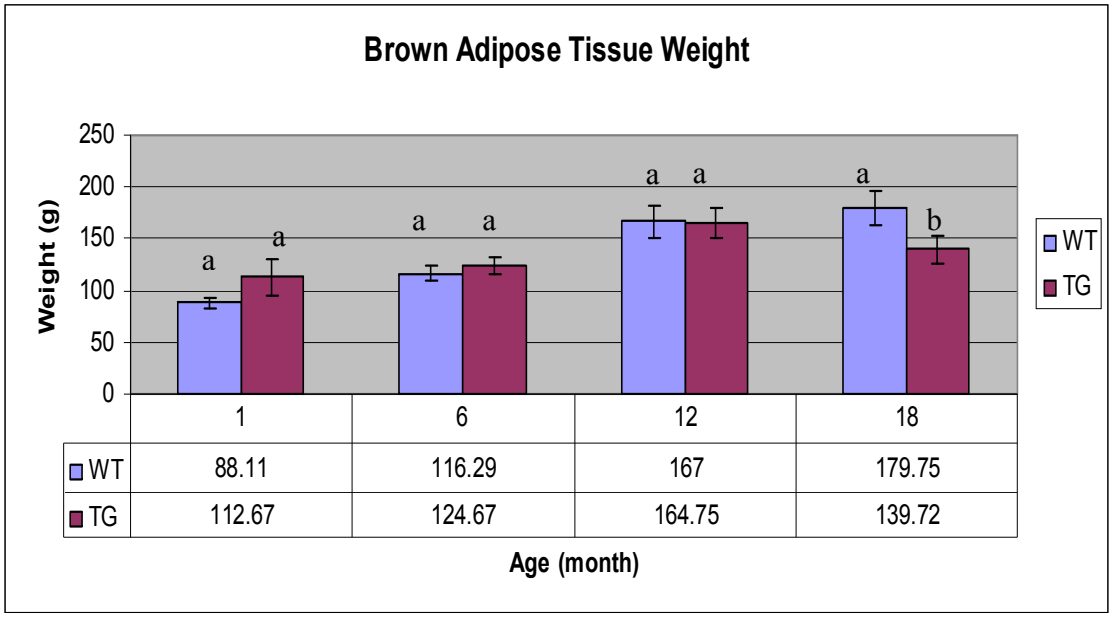


Figure 3.7 Brown adipose tissue weight (g) in wild type and transgenic male mice at four different ages, showing significant reduced weight in transgenic type group at 18 months of age compared to their wild-type littermates. Means with different letters between WT and TG significant at ($P<0.05$).

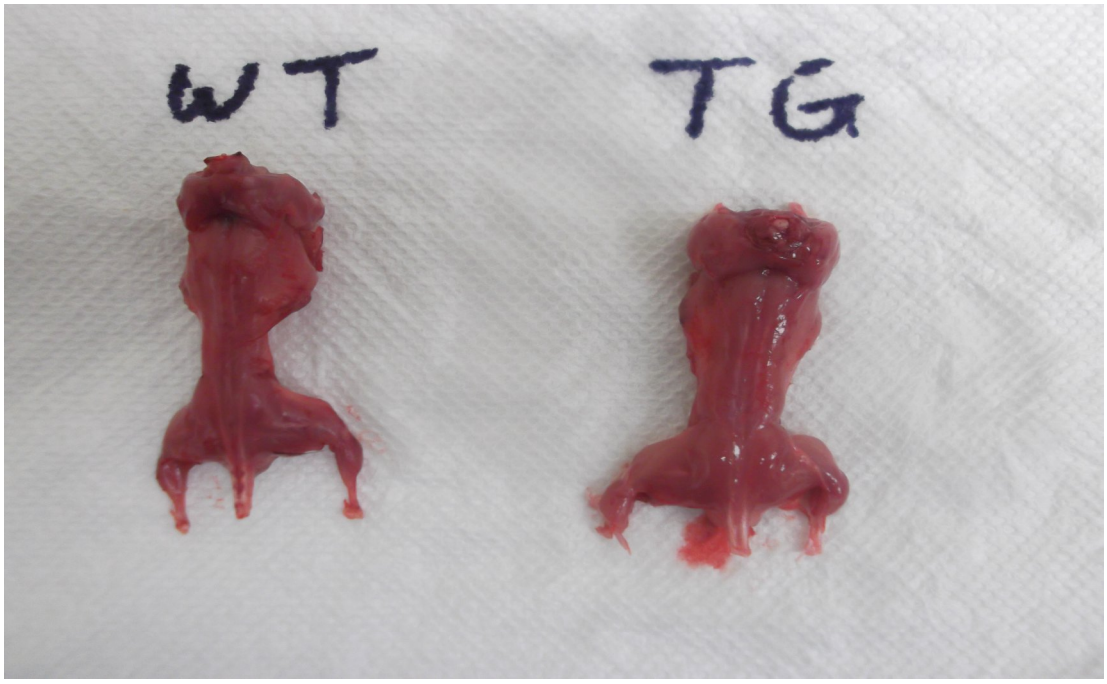
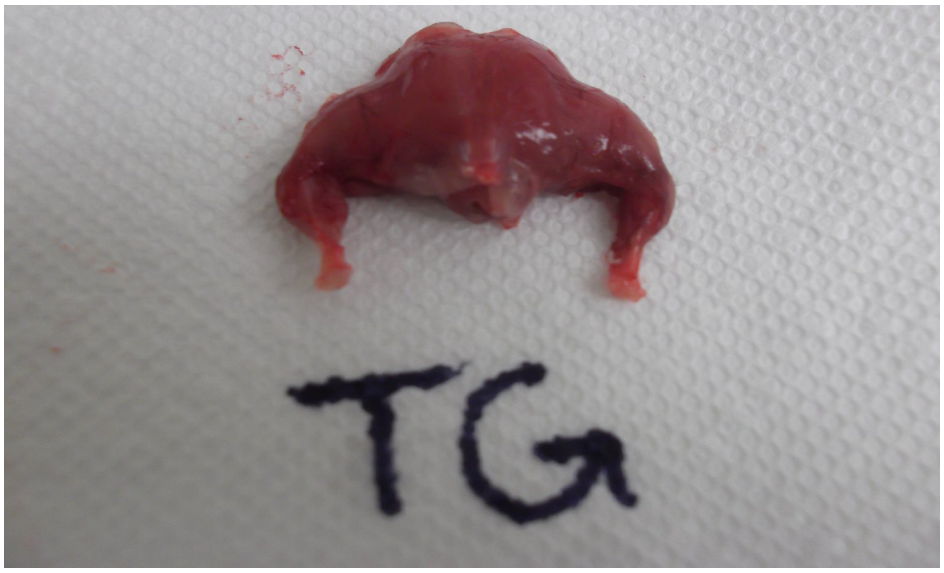


Figure 3.8 Phenotype of transgenic (right side) and littermate control male carcass (left side) at 18 months of mice age. Showing heavier TG male carcass compared to WT male.

A



B

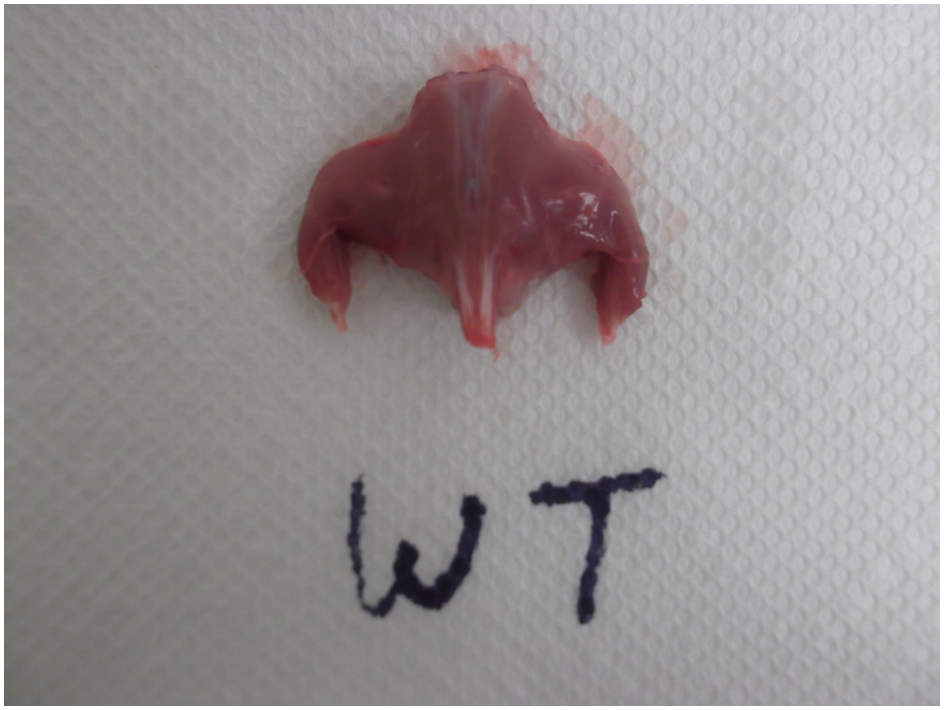


Figure 3.9 Phenotype of TG (**A**) and WT (**B**) hind limbs at 18 months of mice age. Showing heavier TG hind limbs in comparison to WT hind limbs in male.

Figure 3.10 Phenotype of **transgenic** (**left**) and littermate **control** male mice (**right**) dissect showing fat accumulation in wild type aged mice (18 months) compared to transgenic littermate without fat accumulation.

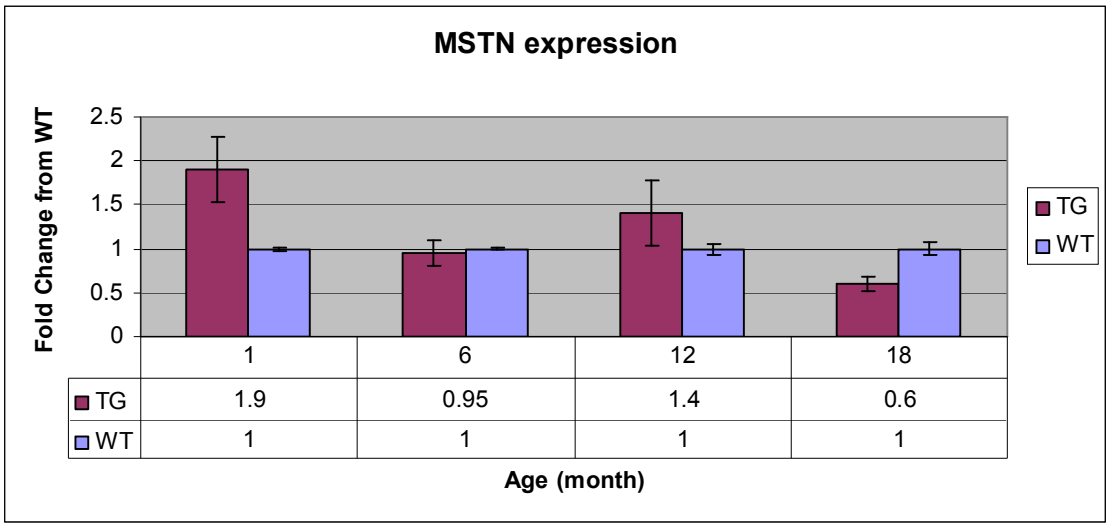


Figure 3.11 Relative fold changes in Myostatin gene expression in transgenic male skeletal muscle compared to their wild-type littermates at different ages, quantified by real time PCR and calculated by $2^{-\Delta\Delta Ct}$.

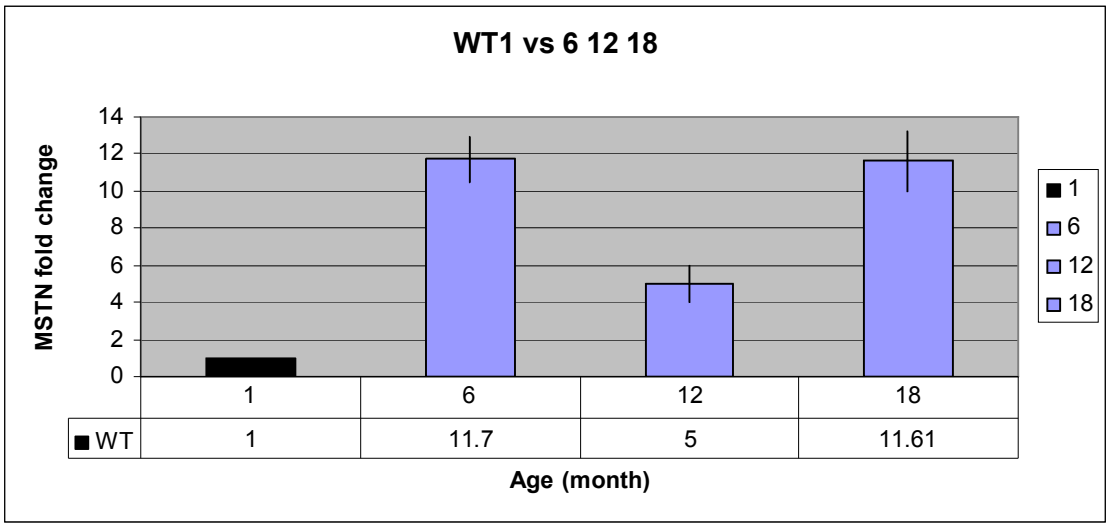


Figure 3.12 Relative fold changes in myostatin gene expression in wild type male skeletal muscle at 6, 12, and 18 months compared to one month of age, quantified by real time PCR and calculated by $2^{-\Delta\Delta Ct}$.

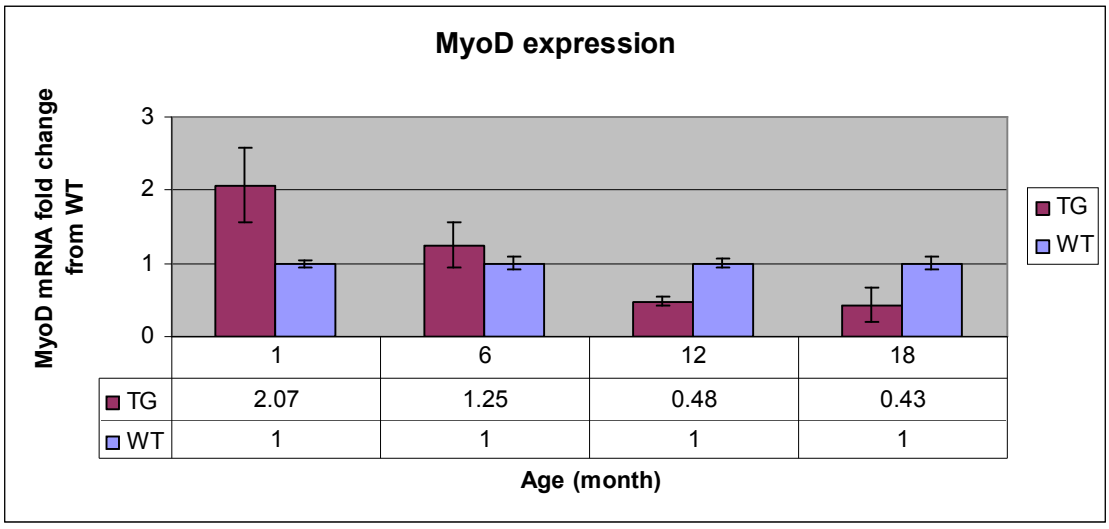


Figure 3.13 Relative fold changes in MyoD gene expression in transgenic male skeletal muscle compared to their wild-type littermates at different ages, quantified by real time PCR and calculated by $2^{-\Delta\Delta Ct}$.

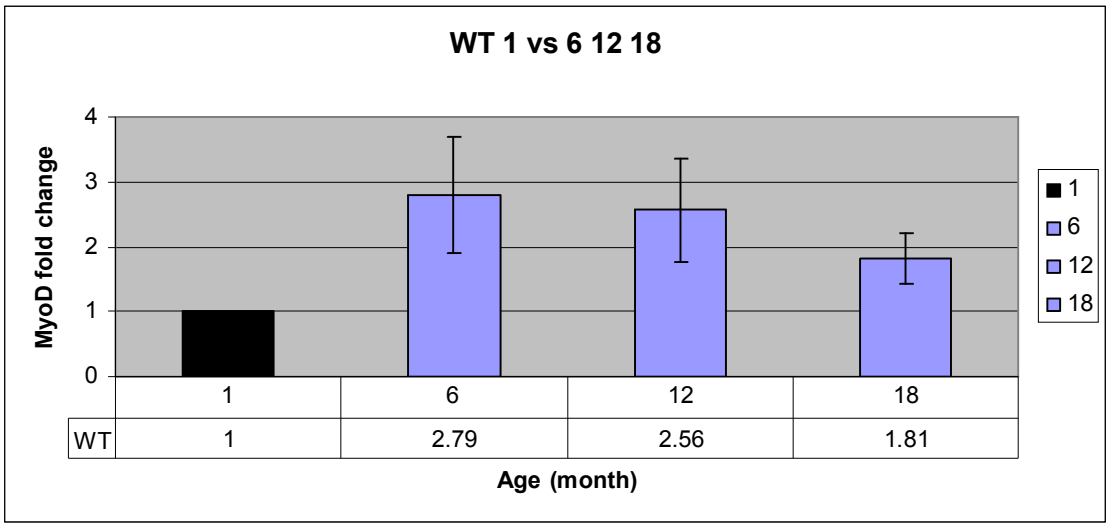


Figure 3.14 Relative fold changes in MyoD gene expression in wild type male skeletal muscle at 6, 12, and 18 months compared to one month of age, quantified by real time PCR and calculated by $2^{-\Delta\Delta Ct}$.

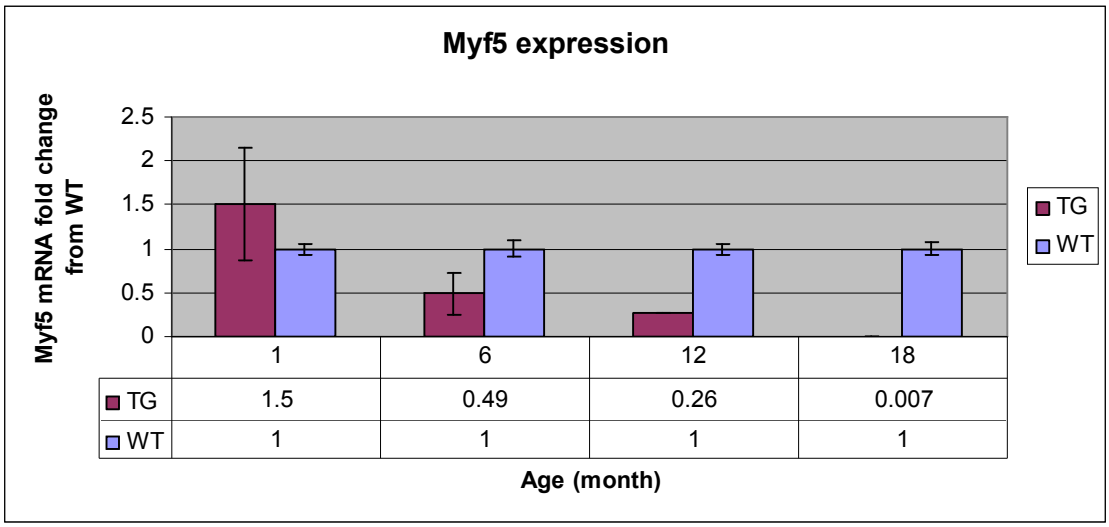


Figure 3.15 Relative fold changes in Myf5 gene expression in transgenic male skeletal muscle compared to their wild-type littermates at different ages, quantified by real time PCR and calculated by $2^{-\Delta\Delta Ct}$.

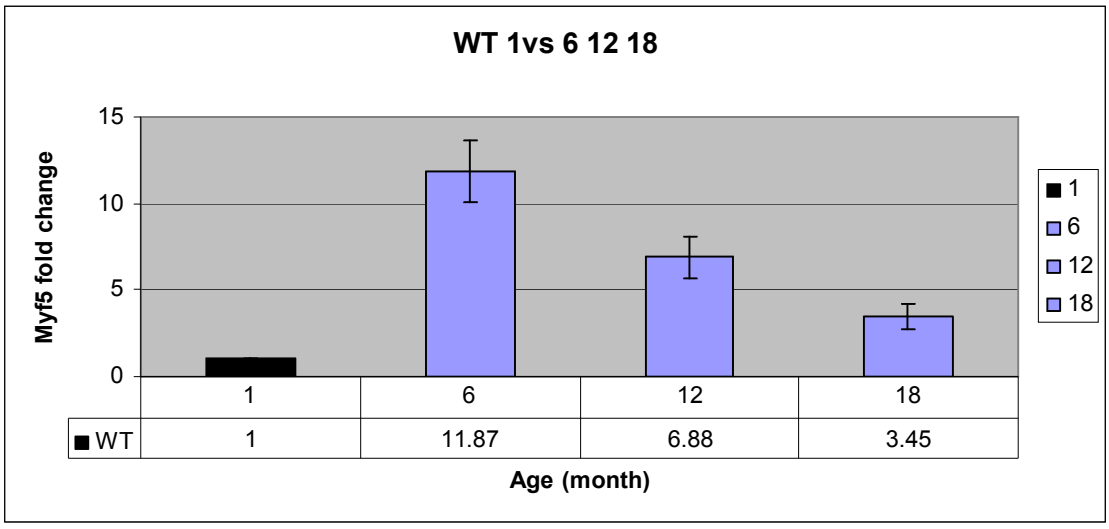


Figure 3.16 Relative fold changes in Myf5 gene expression in wild type male skeletal muscle at 6, 12, and 18 months compared to one month of age, quantified by real time PCR and calculated by $2^{-\Delta\Delta Ct}$.

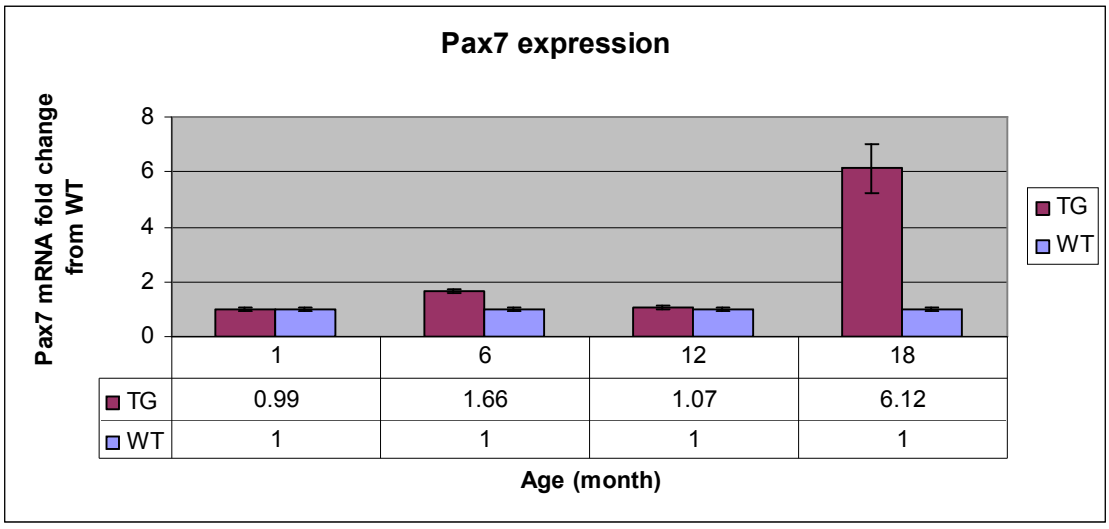


Figure 3.17 Relative fold changes in Pax7 gene expression in transgenic male skeletal muscle compared to their wild-type littermates at different ages, quantified by real time PCR and calculated by $2^{-\Delta\Delta Ct}$.

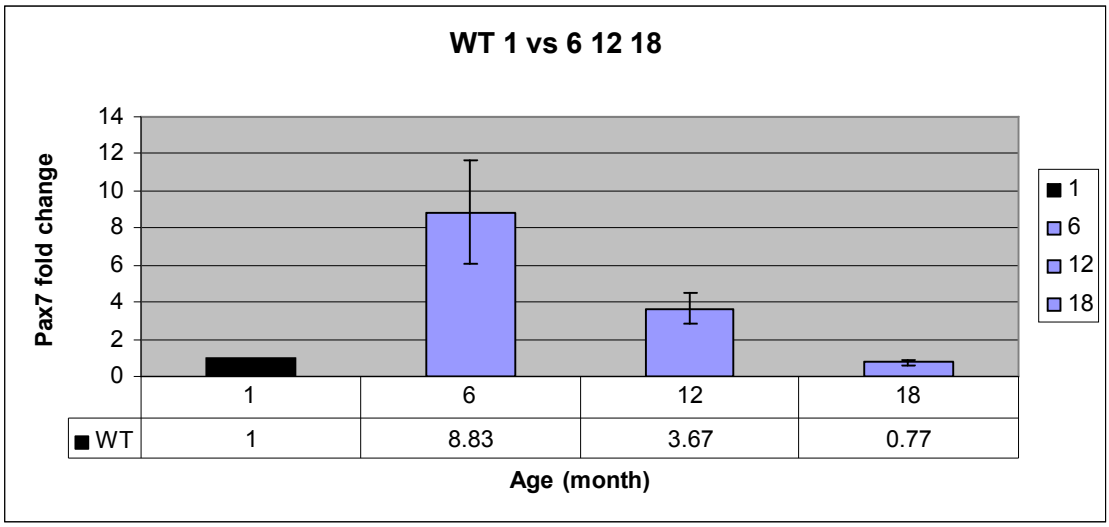


Figure 3.18 Relative fold changes in Pax7 gene expression in wild type male skeletal muscle at 6, 12, and 18 months compared to one month of age, quantified by real time PCR and calculated by $2^{-\Delta\Delta Ct}$.

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

4.1 General discussion

The goals of the current project were to investigate myostatin gene expression in placenta and its role in prenatal muscle growth and placenta weight, also to investigate and better understanding of myostatin gene role in muscle wasting because of age, instead of measuring the expression of other genes related to muscle proliferation and differentiation. Myostatin has been well known as a muscle negative regulator, its expression has been detected in muscle, fat, heart, mammary, uterus, skin and human placenta; there are not enough information about its expression in mice placenta, its level of expression, its role in placenta metabolic transfer of nutrients, moreover its role in fetus and placenta weights. For this reason we have designed an experiment, as it is showed in chapter 2, by using three groups of mice, which include wild (WT), heterozygous transgenic ($MSTN^{TG/+}$), and homozygous transgenic ($MSTN^{TG/TG}$) mice. The results of this experiment showed that myostatin is expressing in placenta and it has more total RNA concentration by twice to three times than muscle tissue. Myostatin inhibition by its propeptide caused significant ($P<0.05$) increase in placenta weight compared to placenta of their wild type littermates, since placenta as an important organ for fetus growth and life, and placenta weight is correlated with dietary intake during pregnancy, so any change in maternal diet will affect the placental-fetal growth and development. Studies have showed that Myostatin can regulate metabolism and glucose uptake in a number of tissues (Antony et al., 2007). It affects glucose uptake independent of insulin and also regulates placental glucose uptake *in vitro* in the human placenta; that increase in placenta weight may due to increase glucose uptake by placenta tissues and then may cause more growth in its cells and tissues.

There is a relatively correlation between placenta weight and fetal weight (Risnes et al., 2009). Placental weight correlates with birth weight (Sibley, 1994). In one study placenta weight was increased in parallel to fetal weight (Jansson et al., 2002), these

results are in agreement with the result of current experiment which increasing placenta weight in transgenic groups was parallel with fetus weights. This increase in fetal weight may due to myostatin role in placental glucose uptake, as other studies showed that myostatin can regulate metabolism and glucose uptake in a number of tissues (Antony et al., 2007). It affects glucose uptake independent of insulin and also regulates placental glucose uptake *in vitro* in the human placenta, here we showed that myostatin inhibition may cause increasing in glucose uptake in mice placenta which consequently increased placenta and fetus weight.

Myostatin gene showed higher expression in both transgenic groups compared to their wild type littermate in both day 10 and 16 of gestation, while its expression was different between wild type and transgenic groups when compared its level between day 10 and 16 of gestation, transgenic group showed reducing its expression, which may caused higher glucose uptake, hence more growth and development in placenta and fetus weight, while wild type group showed increased level of myostatin gene expression in day 16 relatively compared to day 10 of gestation which may cause inhibition of glucose uptake, consequently lower placenta and fetus weights.

A better understanding of skeletal muscle growth, which is important for animal production as well as human health, is important by increasing understanding of physiological processes and molecular pathways associated with skeletal muscle growth and development. Muscle growth is regulated by a group of growth factors; among them, a member of the transforming growth factor-β (TGF-β) super-family, growth differentiation factor 8 (GDF8), which is also known as myostatin, known for its ability to inhibit muscle growth. There are two pathologies of muscle wasting which are of general interest, namely sarcopenia and cachexia (Lenk et al., 2010). A major barrier to effective management of skeletal muscle wasting is the inadequate understanding of its underlying biological mechanisms. Therefore, we designed an experiment to test the hypothesis that myostatin inhibition by over-expression of its propeptide prevents muscle loss and fat accumulation in old age, also to measure the expression level of myostatin and some other genes related to muscle growth and differentiation in an attempt to show better physiological understanding of myostatin and some other genes.

To test the above hypothesis as it is shown in chapter 3, we used male mice at 4 different ages: 1 month (growing), 6 month (young adult), 12 month (middle age) , and 18 month (aged mice) with two groups, wild type and transgenic. They were weighed and sacrificed for muscle tissue dissections and sampling. The results showed that myostatin inhibition caused significant increase ($P<0.05$) in live, carcass and single muscle weight in almost all stages of age, this increase in muscle weight in early life helped the transgenic mice to keep their muscle mass till late of their life or 18 months of age, which helped the transgenic aged mice to reduce the effect of muscle wasting because of age.

Myostatin inhibition has been considered as mean to treat muscular dystrophy, also myostatin null mutation in the mdx-mice showed the improving muscle regeneration (Wanger et al., 2002). It has been shown that myostatin-null mice undergo reduced atrophy during aging along with improved muscle regeneration capacity (Sirriett et al., 2006), illustrating the therapeutic potential of myostatin inhibition to treat sarcopenia. On the other hand, inhibiting myostatin via the administration of an anti-myostatin antibody in aged mice significantly attenuated the decline of skeletal muscle mass and function (LeBrasseur et al., 2009; Murphy et al., 2010). Recovery of muscle mass in the late stages of muscle regeneration is associated with a decrease of myostatin from the elevated levels seen immediately after injury (Kirk et al., 2000). In contrast, myostatin mRNA and/or protein concentrations are increased in skeletal muscle in conditions associated with loss of muscle mass in postnatal life, such as acquired immunodeficiency syndrome (Gonzalez-Cadavid et al., 1998), sarcopenia of old age (Marcell et al., 2001) and disuse atrophy in men under prolonged bed rest (Zachwieja et al., 1999) and in rats during hindlimb unloading (Carlson et al., 1999), after exposure to the microgravity environment of a spaceflight (Lalani et al., 2000), and in association with glucocorticoid induced muscle loss (Lang et al., 2001).

The interactions that occur between skeletal muscle and adipose tissue play a significant role in growth and development which include utilization of energy substrates, muscle growth, and energy storage. The myostatin gene is expressed at low levels in adipose tissue and myostatin protein is found in circulation suggesting that myostatin could have a direct role in regulating adipocyte differentiation or function (Lee, 2004). As it is shown in the current results in chapter 3, transgenic mice showed significant

($P<0.05$) reduce by 80% in adipose tissue content at old age (18 months) compared to wild type littermates, during dissecting the mice, we could easily recognize which mouse is transgenic and which one is wild type, as it is shown in figure 3.10, wild type mice showed a bunch of muscle wasting and fat accumulation in their body at advanced age, whereas transgenic mice keep their muscle mass weight without fat accumulation, this result is in agreement with our previous study (Zhao, et al., 2005) that inhibition of myostatin prevent a high-fat diet from inducing insulin resistance and obesity in mice, suggesting that myostatin-based treatments may be effective in treating diabetes and other metabolic disorder characterized by insulin resistance.

Myostatin expression level increased in transgenic group in all time points relatively compared to wild type mice, its level was fluctuating between high and low through different age.

Myogenic regulatory factors (MRFs) as a transcription factors have role in myogenesis, MyoD and Myf5 are redundant in myoblast specification whereas myogenin with either MyoD or Mrf4 are required for differentiation (Buckingham, 2006). Myostatin known for its role in inhibiting MyoD expression and activity via Smad3, which blocks myoblasts from differentiating into myotubes (Langley et al., 2002) Therefore, myostatin inhibits both myoblast cell proliferation and differentiation. Because myoD is a key regulator of genes involved in the initiation and maintenance of differentiation in muscle, it is likely that myostatin, through MyoD, inhibits the muscle gene expression program. As it is shown in the current experiment (chapter 3) MyoD and Myf5 gene expression increased in transgenic mice in all time points of different age relatively compared to wild type mice. Pax7 gene expression also showed relatively increased level in transgenic mice, transgenic old mice showed very high fold change (6.12 fold) of Pax7 gene which might be the reason of activating, proliferation and differentiation of quiescent satellite cells and fusion to myofibers in transgenic mice. Increasing the expression of Pax7 at old age (18 month) coincided with decreasing of MyoD gene, as it has been mentioned by (McFarlane et al., 2007) that Pax7 inhibit MyoD gene expression.

4.2 Conclusions

In conclusion, research results from this project showed that mouse placenta has 2-3 times more total RNA concentration compared to muscle tissues. Myostatin express in placenta, its level increased with advancing in gestation age in wild-type mice, this result was in contrast to both heterozygous and homozygous transgenic mice which its level decreased with advancing in gestation age. Previous studies showed that myostatin reduces glucose uptake in placenta, our results are a great support to this idea that inhibiting of myostatin activity by its propeptide could provide enough nutrient elements and energy to show heavier weight in transgenic fetuses and placenta.

Myostatin inhibition by its propeptide has a dramatic increase in muscle mass together with continued muscle growth in the old age which could be a novel potential strategy for the treatment of muscle-wasting disorders, such as muscular dystrophy, cachexia and sarcopenia, on the other hand, myostatin inhibition has a significant reduction in fat depots and a depression of adipogenesis, points towards myostatin having a role not only in muscle but also in adipose tissue and this fact could have therapeutic implications in the treatment of obesity.

Based on the observations of gene expression, we believe that myostatin negatively regulate skeletal muscle proliferation and differentiation, and our results clarify that the dramatic effect of myostatin on post-natal growth is primarily due to the ability of myostatin to negatively regulate skeletal muscle satellite cell activation, proliferation and satellite cell self-renewal through its negative regulation on myogenic regulatory factors and Pax7 gene.

4.3 References

Allen E. 1922. The oestrous cycle in the mouse. American Journal of Anatomy 30:297-371.

Amirouche A, Durieux AC, Banzet S, Koulmann N, Bonnefoy R, Mouret C, Bigard X, Peinnequin A, Freyssenet D. 2009. Down-regulation of Akt/mammalian target of rapamycin signaling pathway in response to myostatin overexpression in skeletal muscle. Endocrinology 150:286–294.

Amthor H, Nicholas G, McKinnell I, Kemp CF, Sharma M, Kambadur R, Patel K. 2004. Follistatin complexes Myostatin and antagonises Myostatin-mediated inhibition of myogenesis. Dev Biol 270:19-30.

Amthor H, Otto A, Vulin A, Rochat A, Dumonceaux J, Garcia L, Mouisel E, Hourdé C, Macharia R, Friedrichs M, Relaix F, Zammit PS, Matsakas A, Patel K, Partridge T. 2009. Muscle hypertrophy driven by myostatin blockade does not require stem/precursor-cell activity. Proc Natl Acad Sci USA 106:7479-84.

Antony N, Bass J J, McMahon C D, Mitchel M D. 2007. Myostatin regulates glucose uptake in BeWo cells. Am. J. Physiol. Endocrinol. Metab 293:E1296- E1302.

Artaza, JN, Bhasin S, Magee TR, Reisz-Porszasz S, Shen SR, Groome NP, Meerasahib MF, Gonzalez-Cadavid NF 2005. Myostatin inhibits myogenesis and promotes adipogenesis in C3H IOT(1/2) mesenchymal multipotent cells. Endocrinology 146(8): 3547-57.

Asakura A, Komaki M, Rudnicki M. 2001. Muscle satellite cells are multipotential stem cells that exhibit myogenic, osteogenic, and adipogenic differentiation. Differentiation 68:245-253.

Barker DJ, Gluckman PD, Godfrey KM, Harding JE, Owens JA, Robinson JS.1993. Fetal nutrition and cardiovascular disease in adult life. Lancet. 341:938-941.

Barker DJP, Thornburg K, Osmond C, Kajantie E, Eriksson JG. 2010. The surface area of the placenta and hypertension in the offspring in later life. Int J Dev Biol. 54:525-30.

Barker DJP. 2006. The developmental origins of insulin resistance. Horm. Res. 64:2-7.

Beauchamp JR, Heslop L, Yu DS, Tajbakh S. Kelly RG, Wernig A, Buckingham ME, Partridge TA, Zammit PS. 2000. Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells. J. Cell Biol. 151:1221-1234.

Belkacemi L, Nelson DM, Desai M, Ross MG. 2010. Maternal undernutrition influences placental-fetal development. Biology of Reproduction. 83:325-331.

Bischoff R, Heintz C. 1994. Enhancement of Skeletal-Muscle Regeneration. Dev Dyn 201:41-54.

Bischoff R. 1989. Analysis of muscle regeneration using single myofibers in culture. Med. Sci. Sports Exerc. 21:164-172.

Bo¨ttinger, E P, Factor VM, Tsang MLS, Weatherbee JA, Kopp JB, Qian SW, Wakefield LM, Roberts AB, Thorgeirsson SS, and Sporn MB. 1996. The recombinant proregion of transforming growth factor β1 (latency-associated peptide) inhibits active transforming growth factor β1 intransgenic mice. Proc. Natl. Acad. Sci. USA 93:5877–5882.

Boettiger D, Enomoto-Iwamoto M, Yoon H Y, Hofer U, Menko AS, Chiquet-Ehrismann R. 1995. Regulation of integrin a5b1 affinity during myogenic differentiation. Dev. Biol 169:261–272.

Bogdanovich S, Krag TOB, Barton ER, Morris LD, Whittemore LA, Ahima RS, Khurana TS. 2002. Functional improvement of dystrophic muscle by myostatin blockade. Nature A-Z Index 420: 418-421.

Bogdanovich S, Perkins KJ, Krag TO, Whittemore LA, Khurana TS. 2005. Myostatin propeptide-mediated amelioration of dystrophic pathophysiology. FASEB J. 19:543–549.

Braun T, Arnold HH. 1995. Inactivation of Myf-6 and Myf-5 genes in mice leads to alterations in skeletal muscle development. The European Molecular Biology Organization (EMBO) Journal 14:1176-1186.

Braun T, Rudnicki MA, Arnold HH, Jacenisch R. 1992. Targeted inactivation of the mouse regulatory gene Myf5 results in abnormal distal rib development and early postnatal death in homozygous mouse mutants. Cell 71:369-382.

Brooke MH, Kaiser K K. 1970. Three 'myosin adenosine triphosphatase' systems: the nature of their pH lability and sulphydryl dependence. Journal of Histochemistry and Cytochemistry 18:670-672.

Buckingham M. 2006. Myogenic progenitor cells and skeletal myogenesis in vertebrates. Curr Opin Genet Dev. 16:525-532.

Bustin S. 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): Trends and problems. Journal of Molecular Endocrinology 29:23-39.

Carlson CJ, Booth FW, Gordon SE. 1999. Skeletal muscle myostatin mRNA expression is fiber-type specific and increases during hindlimb unloading. American Journal of Physiology- Regulatory, Integrative and Comparative Physiology 277:601-606.

Chelh I, Meunier B, Picard B, Reecy JM, Chevalier C, Hocquette JF, Cassar-Malek I. 2009. Molecular profiles of quadriceps muscle in myostatin-null mice reveal PI3K and apoptotic pathways as myostatin targets. BMC Genomics 10:196.

Chen J, Goldhamer D. 2003. Skeletal muscle stem cells. Reproductive Biology and Endocrinology, 1:101-108.

Clop A, Marcq F, Takeda H, Pirottin D, Tordoir X, Bibé B, Bouix J, Caiment F, Elsen JM, 70 Eychenne F, Larzul C, Laville E, Meish F, Milenkovic D, Tobin J, Charlier C, Georges M. 2006. A mutation creating a potential illegitimate microRNA arget site in the myostatin gene affects muscularity in sheep. Nat Genet 38:813-818.

Cole LA. 2009. New discoveries on the biology and detection of human chorionic gonadotropin. Reprod. Biol. Endocrinology 7:8. doi:10.1186/1477-7827-7 8.PMC 2649930. PMID 19171054.

Cornelison DD, Olwin BB, Rudnicki MA, Wold BJ. 2000. MyoD−/− satellite cells in single-fiber culture are differentiation defective and MRF4 deficient. Developmental Biology 224:122-137.

Cornelison DD. Wold BJ. 1997 Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. Developmental Biology 191:270-283.

David JD, See WM, Higginbotham CA. 1981. Fusion of chick embryo skeletal myoblasts: Role of calcium influx preceding membrane union. Developmental Biology 82: 297–307.

Dirks A, Leeuwenburgh C. 2002. Apoptosis in skeletal muscle with aging. Am. J. Physiol. Regul. Integr. Comp. Physiol. 282:R519–R527

Faulkner JA, Brooks SV, Zerba E. 1995. Muscle atrophy and weakness with aging: contraction-induced injury as an underlying mechanism. J. Gerontol. A Biol. Sci. Med. Sci. 50:124–129.

Faulkner JA, Larkin LM, Claflin DR, Brooks SV. 2007. Age-related changes in the structure and function of skeletal muscles. Clin. Exp. Pharmacol. Physiol. 34: 1091–1096.

Feldman BJ, Streeper RS, Farese RV, Jr, Yamamoto KR. 2006. Myostatin modulates adipogenesis to generate adipocytes with favorable metabolic effects. Proceeding of the National Academy of Sciences of the United States of America. 103(42): 15675-15680.

Fiedler F, Lemon MJC, Hirschauer C, Leysath G, Lottspeich F, Henschen A, Gau W, Bhoola KD. 1983. Purification and properties of Guinea-Pig submandibular gland kallikrein. Biochem J 209:125-134.

Florini JR, Ewton DZ, Coolican SA. 1996. Growth hormone and the insulin-like growth factor system in myogenesis. Endocrin Rev 17:481-517.

Gauthier GF. 1969. On the relationship of ultrastructural and cytochemical features to color in mammalian skeletal muscle. Zeitschrift fur Zellforschung 95: 462-482.

Godfrey KM. 2002. The role of the placenta in fetal programming. Placenta. 23 (Suppl A):S20-7.

Gonzalez-Cadavid N, Taylor WE, Yarasheski K, Sinha-Hikim, I, Ma K, Ezzat S, Shen R, Lalani R, Asa S, Mamita M, Nair G, Arver S, Bhasin S. 1998. Organization of the human myostatin gene and expression in healthy men and HIV-infected men with muscle wasting. Proceedings of the National Academy of Sciences, 95:14938-14943.

Greenwald GS, Rothchild I. 1968. Formation and maintenance of corpora lutea in laboratory animals. J Anim Sci 27 Suppl 1:139-162.

Grobet L, Martin LJ, Poncelet D, Pirottin D, Brouwers B, Riquet J, Schoeberlein A, Dunner S, Ménissier F, Massabanda J, Fries R, Hanset R, Georges M. 1997. A deletion in the bovine myostatin gene causes the double-muscled phenotype in cattle. Nature Genet 17:71–74.

Grounds MD, Yablonka-Reuveni Z. 1993. Molecular and cell Biology of skeletal muscle regeneration. Mol. Cell Biol. Hum. Dis. Ser. 3:210-256.

Hadjipavlou G, Matika O, Clop A, Bishop SC. 2008. Two single nucleotide polymorphisms in the myostatin (GDF8) gene have significant association with muscle depth of commercial Charollais sheep. Anim Genet 39:346-353.

Hamrick MW, Shi X, Zhang W, Pennington C, Thakore H, Haque M, Kang B, Isales CM, Fulzele S, Wenger KH. 2007. Loss of myostatin (GDF8) function increases osteogenic differentiation of bone marrow-derived mesenchymal stem cells but the osteogenic effect is ablated with unloading. Bone 40:1544-1553.

Hanrahan JP, Eisen EJ, Lagates JE. 1973. Effects of population size and selection intensity of short-term response to selection for postweaning gain in mice. Genet 73:513-530.

Hasty P, Bradley A, Morris JR, Edmondson DG, Venuti JM, Olson EN, Klein WH. 1993. Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. Nature 364:501-506.

Hawke TJ, Garry DJ. 2001. Myogenic satellite cells: physiology to molecular biology. J Appl Physiol 91:534-551.

Heid CA, Stevens J, Livak KJ, Williams PM. 1996. Real time quantitative PCR. Genome Research 6:986-994.

Hemachandra AH, Klebanoff MA, Duggan AK, et al. 2006. The association between intrauterine growth restriction in the full-term infant and high blood pressure at age 7 years: results from the collaborative perinatal project. Int J Epidemiol. 35:871-877.

Higuchi R, Fockler C, Dollinger G, Watson R. 1993. Kinetic PCR analysis: Real-time monitoring of DNA amplification reactions. Biotechnology 11:1026-1030.

Hill JJ, Davies MV, Pearson AA, Wang JH, Hewick RM, Wolfman NM, Qiu YC. 2002. The myostatin propeptide and the follistatin-related gene are inhibitory binding proteins of myostatin in normal serum. J Biol Chem 277:40735-40741.

Hill JJ, Qiu Y, Hewick RM, Wolfman NM. 2003. Regulation of myostatin *in vivo* by growth and differentiation factor-associated serum protein-1: a novel protein with protease inhibitor and follistatin domains. Mol Endocrinol 17:1144–1154.

Holland PM, Abramson RD, Watson R, Gelfand DH. 1991. Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of thermus aquaticus DNA polymerase. Proceedings of the National Academy of Sciences 88:7276-7280.

Hoodless PA, Wrana JL. 1998. Mechanism and function of signaling by the TGF beta superfamily. Curr Top Microbiol Immunol 228:235-272.

Hosoyama T, Yamanouchi K, Nishihara M. 2006. Role of serum myostatin during the lactation period. J Reprod Dev 52:469-478.

Hu X, Guo H, He Y, Wang S, Zhang L, Huang X, Roy SW, Lu W, Hu J, Bao Z. 2010. Molecular characterization of Myostatin gene from Zhikong scallop Chlamys farreri (Jones et Preston 1904). Genes Genet Syst 85:207-218.

Jansson T, Ekstrand Y, Bjorn C, Wennergren M, Powell TL. 2002. Alterations in the activity of placental amino acid transporters in pregnancies complicated by diabetes. Diabetes 51:2214-9.

Ji M, Zhang Q, Ye J, Wang X, Yang W, Zhu D. 2008. Myostatin induces p300 degradation to silence cyclin D1 expression through the PI3K/PTEN/Akt pathway. Cell Signal 20:1452-8.

Ji S, Losinski RL, Cornelius SG, Frank GR, Willis GM, Gerrard DE, Depreux FF, Spurlock ME. 1998. Myostatin expression in porcine tissues: tissue specificity and developmental and postnatal regulation. Am J Physiol 275:R1265-1273.

Jin HJ, Dunn MA, Borthakur D, Kim YS. 2004. Refolding and purification of unprocessed porcine myostatin expressed in Escherichia coli. Protein Expr Purif 35:1-10.

Kambadur R, Sharma M, Smith TP, Bass JJ. 1997. Mutations in myostatin (GDF8) in double-muscled Belgian Blue and Piedmontese cattle. Genome Res 7:910-916.

Kawano F, Takeno Y, Nakai N, Higo Y, Terada M, Ohira T, Nonaka I, Ohira Y. 2008. Essential role of satellite cells in the growth of rat soleus muscle fibers. Am J Physiol Cell Physiol 295:C458-467.

Kim YS, Bobbili NK, Lee YK, Jin HJ, Dunn MA. 2007. Production of a polyclonal anti-myostatin antibody and the effects of in ovo administration of the antibody on posthatch broiler growth and muscle mass. Poult Sci 86:1196-205.

Kim, HS, Liang L, Dean RG, Hausman DB, Hartzell DL, Baile CA. 2001. Inhibition of preadipocyte differentiation by myostatin treatment in 3T3-Ll cultures. Biochem Biophvs Res Commun 281: 902-6.

Kirk S, Oldham J, Kambadur R, Sharma M, dobbie P, Bass J. 2000. Myostatin regulation during skeletal muscle regeneration. J Cell Physiol 184:356-363.

Kishioka Y, Thomas M, Wakamatsu J, Hattori A, Sharma M, Kambadur R, Nishimura T. 2008. Decorin enhances the proliferation and differentiation of myogenic cells through suppressing myostatin activity. J Cell Physiol 215:856-867.

Klont RE, Brocks L, Eikelenboom G. 1998. Muscle fiber type and meat quality. Meat Science 49:S219–S229.

Knudsen K A. 1985. The calcium-dependent myoblast adhesion that precedes cell fusion is mediated by glycoproteins. J. Cell Biol 101: 891–897.

Kolodziejczyk SM, Hall BK. 1996. Signal transduction and TGF-beta superfamily receptors. Biochem Cell Biol 74:299-314.

Kuang S, Kuroda K, Le-Grand F, Rudnicki MA. 2007. Asymmetric self-renewal and commitment of satellite stem cells in muscle. Cell 129:999-1010.

Kuruvilla AG, D`Souza SW, Glazier JD, Mahendran D, Maresh MJ. 1994Altered activity of the system A amino acid transporter in microvillous membrane vesicle from placentas of macrosomic babies born to diabetic women. Journal of Clinical Investigation 94:689-95.

Lalani R, Bhasin S, Byhower F, Tranuzzer R, Grant M, Shen R, Asa S, Ezzat S, Gonzalez-Cadavid NF. 2000. Myostatin and isulin-like growth factor-I and –II expression in the muscle of rats exposed to the microgravity environment of the NeuroLab space shuttle flight. Endocrinology 167:417-428.

Lang CH, Silvis C, Nystrom G, Frost RA. 2001. Regulation of myostatin by glucocorticoids after thermal injury. FASEB J 15:1807-1809.

Langley B, Thomas M, Bishop A, Sharma M, Gilmour S, Kambadur R. 2002. Myostatin inhibits myoblast differentiation by down-regulating MyoD expression. The Journal of Biological Chemistry 277:49831–49840.

LeBrasseur NK, Schelhorn TM, Bernardo BL, Cosgrove PG, Loria PM, Brown TA. 2009. Myostatin inhibition enhances the effects of exercise on performance and metabolic outcomes in aged mice. J. Gerontol. A Biol. Sci. Med. Sci. 64:940-948.

Lee S-J, McPherron AC. 2001. Regulation of myostatin activity and muscle growth. Proc Natl Acad Sci USA 98:9306–9311.

Lee S-J. 2004. Regulation of muscle mass by myostatin. Annu Rev Cell Dev Biol 20: 61–86.

Lee SJ. 2008. Genetic Analysis of the Role of Proteolysis in the Activation of Latent Myostatin. PLoS One 3:e1628.

Lee, SJ. & McPherron, A.C. 2001. Regulation of myostatin activity and muscle growth. Proc. Natl. Acad. Sci. USA 98:9306-9311.

Léger B, Derave W, De Bock K, Hespel P, Russell AP. 2008. Human sarcopenia reveals an increase in SOCS-3 and myostatin and a reduced efficiency of Akt phosphorylation. Rejuvenation Res. 11:163B-175B.

Lenk K, Schuler G, Adams V. 2010. Skeletal muscle wasting in cachexia and sarcopenia: molecular pathophysiology and impact of exercise training. J Cachexia Sarcopenia Muscle 1:9–21.

Li Z, Zhao B, Kim YS, Hu CY, Yang J. 2010. Administration of a mutated myostatin propeptide to neonatal mice significantly enhances skeletal muscle growth. Mol Reprod Dev 77:76-82.

Lin J, Arnold HB, Della-Fera MA, Azain MJ, Hartzell DL, Baile CA. 2002. Myostatin knockout in mice increases myogenesis and decreases adipogenesis. Biochem Biophvs Res Commun 291: 701-6.

Listrat A, Hocquette JF, Picard B, Menissier F, Djiane J, Jammes H. 2005. Growth hormone receptor gene expression in the skeletal muscle of normal and double muscled bovines during foetal development. Reprod. Nutr. Dev. 45:393-403.

Liu CM, Yang Z, Liu CW, Wang R, Tien P, Dale R, Sun LQ. 2008. Myostatin antisense RNA-mediated muscle growth in normal and cancer cachexia mice. Gene Ther. 15:155–160.

Liu D, Black BL, Derynck R. 2001. TGF-beta inhibits muscle differentiation through functional repression of myogenic transcription factors by Smad3. Genes Dev 15:2950-2966.

Lowe DA, Alway SE. 1999. Stretch-induced myogenin, MyoD, and MRF4 expression and acute hypertrophy in quail slow-tonic muscle are not dependent upon satellite cell proliferation. Cell Tissue Res 296:531-539.

Luff RA, Goldspink G. 1970. Total number of fibers in muscles of several strains of mice. J Anim Sci 30: 891-893.

Lynch GS. 2004. Tackling Australia's future health problems: developing strategies to combat sarcopenia-age-related muscle wasting and weakness. Intern. Med. J. 34:294–296.

Lyons JA, Haring JS, Biga PR. 2010. Myostatin expression, lymphocyte population, and potential cytokine production correlate with predisposition to high-fat diet induced obesity in mice. PLoS One 5:e12928.

Maccatrozzo L, Bargelloni L, Radaelli G, Mascarello F, Patarnello T. 2001. Characterization of the myostatin gene in the gilthead seabream (Sparus aurata): sequence, genomic structure, and expression pattern. Mar Biotechnol (NY) 3:224-230.

Manickam R, Pena RN, Whitelaw CB. 2008. Mammary gland differentiation inversely correlates with GDF-8 expression. Mol Reprod Dev 75: 1783-1788.

Marcell TJ, Harman SM, Urban RJ, Metz DD, Rodregers BD, Blackman MR. 2001. Comparison of GH, IGF-I, and testosterone with mRNA of receptors and myostatin in skeletal muscle in older men. Am J Physiol Endocrinol Metab 281:E1159-E1164.

Maroto M, Reshef R, Munsterberg AE, Koester S, Goulding M, Lassar AB. 1997. Ectopic Pax-3 activates MyoD and Myf-5 expression in embryonic mesoderm and neural tissue. Cell 89:139-148.

Massague J. 1998. TGF-beta signal transduction. Annu Rev Biochem 67:753-791.

Matsakas A, Foster K, Otto A, Macharia R, Elashry MI, Feist S, Graham I, Foster H, Yaworsky P, Walsh F, Dickson G, Patel K. 2009. Molecular, cellular and physiological investigation of myostatin propeptide-mediated muscle growth in adult mice. Neuromuscul Disord 19:489-499.

McCrabb GJ, Egan AR, Hosking BJ. 1991. Maternal undernutrition during mid-pregnancy in sheep. Placental size and its relationship to calcium transfer during late pregnancy. Br J Nutr. 65:157-168.

McCroskery S, Thomas M, Maxwell L, Sharma M, Kambadur R. 2003. Myostatin negatively regulates satellite cell activation and self-renewal. J Cell Biol, 162:1135-1147.

McCroskery S, Thomas M, Platt L, Hennebry A, Nishimura T, McLeary L, Sharma M, and Kambadur R. 2005. Improved muscle healing through enhanced regeneration and reduced fibrosis in myostatin-null mice. J Cell Sci. 118(Pt15):3531-3541.

McFarlane C, Hennebry A, Thomas M, Plummer E, Ling N, Sharma M, Kambadur R. 2008. Myostatin signals through Pax7 to regulate satellite cell self-renewal. Experimental Cell Research 314:317-329.

McFarlane C, Plummer E, Thomas M, Hennebry A, Ashby M, Ling N, Smith H, Sharma M, Kambadur R. 2006. Myostatin induces cachexia by activating the ubiquitin proteolytic system through an NF-kappaB-independent, FoxO1-dependent mechanism. J Cell Physiol 209:501-14.

McKinnell IW, Parise G, Rudnicki, MA. 2005. Muscle stem cells and regenerative myogenesis. In Current Topics in Developmental Biology (113-130) Gerald P. Schatten (Editor) Academic Press.

McPherron AC, Lawler AM, Lee S-J. 1997. Regulation of skeletal muscle mass in mice by a new TGF-ß superfamily member. Nature 387:83–90.

McPherron AC, Lee S-J. 1997. Double muscling in cattle due to mutations in the myostatin gene. Proc Natl Acad Sci USA 94:12457–12461.

McPherron AC, Lee SJ. 2002. Suppression of body fat accumulation in myostatin-deficient mice. J Clin Invest 109:595-601.

Megeney LA, Rudnicki MA. 1995. Determination versus differentiation and the MyoD family of transcription factors. Biochemistry and Cell Biology 73:723-732.

Miller JB, Schaefer L, Dominov JA. 1999. Seeking muscle stem cells. Curr Top Develop Biol, 43:191-219.

Mitchell MD, Osepchook CC, Leung KC, McMahon D, Bass JJ. 2006. Myostatin is a human placental product that regulates glucose uptake. The Journal of Clinical Endocrinology & Metabolism 91(4): 1434-1437.

Miura T, Kishioka Y, Wakamatsu J, Hattori A, Hennebry A, Berry CJ, Sharma M, Kambadur R, Nishimura T. 2006. Decorin binds myostatin and modulates its activity to muscle cells. Biochem Biophys Res Commun 340:675-680.

Mosher DS, Quignon P, Bustamante CD, Sutter NB, Mellersh CS, Parker HG, Ostrander EA. 2007. A mutation in the myostatin gene increases muscle mass and enhances racing 79 performance in heterozygote dogs. PLoS Genet 3:0779-0786.

Murphy KT, Ryall JG, Snell SM, Nair L, Koopman R, Krasney PA, Ibebunjo C, Holden KS, Loria PM, Salatto CT, Lynch GS. 2010. Antibody-directed myostatin inhibition improves diaphragm pathology in young but not adult dystrophic mdx mice. Am. J. Pathol. 176:2425–2434.

Nabeshima Y, Hanaoka K, Hayasaka M, Esumi E, Li S, Nonaka I, Nabeshima Y. 1993. Myogenin gene disruption results in perinatal lethality because of severe muscle defect. Nature 364:532-535.

Nakatani M, Takehara Y, Sugino H, Matsumoto M, Hashimoto O, Hasegawa Y, Murakami T, Uezumi A, Takeda S, Noji S, Sunada Y, Tsuchida K. 2008. Transgenic expression of a myostatin inhibitor derived from follistatin increases skeletal muscle mass and ameliorates dystrophic pathology in mdx mice. FASEB J 22:477-487.

Nicholas G, Thomas M, Langley B, Somers W, Patel K, Kemp CF, Sharma M, Kambadur R. 2002. Titin-cap associates with, and regulates secretion of, myostatin. J Cell Physiol 193:120–131.

Nishimura T, Oyama K, Kishioka Y, Wakamatsu J, Hattori A. 2007. Spatiotemporal expression of decorin and myostatin during rat skeletal muscle development. Biochem Biophys Res Commun 361:896-902.

Niswender GD, Juengel JL, Silva PJ, Rollyson /mk McIntush EW. 2000. Mechanisms controlling the function and life span of the corpus luteum. Physiol Rev 80:1-29.

O'Brien SP, Seipel K, Medley QG, Bronson R, Segal R, Streuli M. 2000. Skeletal muscle deformity and neuronal disorder in Trio exchange factor-deficient mouse embryos. Proc Natl Acad Sci USA 97:12074-8.

O'Connor RS, Pavlath GK, McCarthy JJ, Esser KA. 2007. Last Word on Point:Counterpoint: Satellite cell addition is/is not obligatory for skeletal muscle hypertrophy. J Appl Physiol 103:1107.

Oksbjerg N, Gondret F, Vestergaard M. 2004. Basic principles of muscle development and growth in meat-producing mammals as affected by the insulin-like growth factor (IGF) system. Domest Anim Endocrinol 27:219-40.

Olguin HC, Olwin BB. 2004. Pax7 up-regulation inhibits myogenesis and cell cycle progression in satellite cells: a potential mechanism for self-renewal. Dev. Biol. 275:375-388.

Ontell M, Kozeka K. 1984. The organogenesis of murine striated muscle: a cytoarchitectural study. Am J Anat 171:133-148.

Ordahl CP, Ledouarin NM. 1992. Two myogenic lineages within the developing somite. Develop 114:339-353.

Patruno M, Caliaro F, Maccatrozzo L, Sacchetto R, Martinello T, Toniolo L, Reggiani C, Mascarello F. 2008. Myostatin shows a specific expression pattern in pig skeletal and extraocular muscles during pre- and post-natal growth. Differentiation 76:168-181.

Perry R, Rudnick M. 2000. Molecular mechanisms regulating myogenic determination and differentiation. Front Biosci 5:D750-67.

Peter JB, Bainard RJ, Edgerton VR, Gillespie CA, Stempel KE. 1972. Metabolic profiles of three fiber types of skeletal muscle in guinea pigs and rabbits. Biochemistry 11:2627-2633.

Peters IR, Helps CR, Hall EJ, Day MJ. 2004. Real-time RT-PCR: considerations for efficient and sensitive assay design. J Immunol Methods 286(1-2):203-17.

Pirottin D, Grobet L, Adamantidis A, Farnir F, Herens C, Daa Schrøder H, Georges M. 2005. Transgenic engineering of male-specific muscular hypertrophy. Proc Natl Acad Sci U S A. 102:6413-8.

Powell SE, Aberle ED. 1981. Skeletal muscle and adipose tissue cellularity in runt and normal birth weight swine. J Anim Sci 52:748-756.

Rebbapragada A, Benchabane H, Wrana JL, Celeste AJ, Attisano L. 2003. Myostatin signals through a transforming growth factor beta-like signaling pathway to block adipogenesis. Mol Cell Biol 23:7230-7242.

Reecy JM, Spurlock DM, Stahl CH. 2006. Gene expression profiling: Insights into skeletal muscle growth and development. J ANIM SCI 84:E150-E154.

Rehfeldt C, Fiedler I, Dietl G, Ender K. 2000. Myogenesis and postnatal skeletal muscle cell growth as influenced by selection. Livest Prod Sci 66:177-188.

Reisz-Porszasz, Bhasin S, Artaza JN, Shen R, Sinha- Hikim I, Hogue A, Fielder TJ, Gonzalez-Cadavid NF. 2003. Lower skeletal muscle mass in male transgenic mice with muscle-specific over-expression of myostatin. Am J Physiol Endocrinol Metab. 285:E876-E888.

Rios R, Carneiro I, Arce VM, Devesa J. 2002. Myostatin is an inhibitor of myogenic differentiation. Am J Physiol Cell Physiol 282:C993–C999.

Risnes KR, Romundstad PR, Nilsen TIL, Eskild A, Vatten LJ. 2009. Placental weight relative to birth weight and long-term cardiovascular mortality: findings from cohort of 31,307 men and women. Am J Epidemiol. 170: 622-631.

Rodgers BD, Garikipati DK. 2008. Clinical, agricultural, and evolutionary biology of myostatin: a comparative review. Endocr Rev 29:513-534.

Rodgers BD, Weber GM. 2001. Sequence conservation among fish myostatin orthologues and the characterization of two additional cDNA clones from Morone saxatilis and Morone americana. Comp Biochem Physiol B Biochem Mol Biol 129:597-603.

Rodino-Klapac LR, Haidet AM, Kota J, Handy C, Kaspar BK, Mendell JR. 2009. Inhibition of myostatin with emphasis on follistatin as a therapy for muscle disease. Mus Ner 39:283-296.

Rosenblatt JD, Woods RI. 1992. Hypertrophy of Rat Extensor Digitorum Longus Muscle Injected with Bupivacaine - a Sequential Histochemical, Immunohistochemical, Histological and Morphometric Study. J Anat 181:11-27.

Rossant J, Cross JC. 2001. Placental development: lessons from mouse mutants. Nat Rev Genet 2:538-48.

Roubenoff R, Rall LC, Veldhuis JD, Kehayias JJ, Rosen C, Nicolson M, Lundgren N, and Reichlin S. 1998. The relationship between growth hormone kinetics and sarcopenia in postmenopausal women: the role of fat mass and leptin.J. Clin. Endocrinol. Metab. 83:1502–1506.

Rowe RW, Goldspink G. 1969. Muscle fiber growth in five different muscles in both sexes of mice. J Anat 104:519-530.

Rudnicki MA, Braun T, Hinuma S, Jaenisch R. 1992. Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development. Cell 71:383-390.

Rudnicki MA, Jaenisch R. 1995. The Myod family of transcription factors and skeletal myogenesis. Bioes 17:203-209.

Rudnicki MA, Schnegelsberg PN, Stead RH, Braun T, Arnold RH, Jaenisch R. 1993. MyoD or Myf-5 is required for the formation of skeletal muscle. Cell 75:1351-1359.

Rugh, R. 1968. The Mouse: Its Reproduction and Development. Oxford University Press, New York.

Salafia CM, Charles AK, Maas EM. 2006. Placental and fetal growth restriction. Clin Obstet Gynecol. 49:236-256.

Saremi A, Gharakhanloo R, Sharghi S, Gharaati MR, Larijani B, Omidfar K. 2010. Effects of oral creatine and resistance training on serum myostatin and GASP-1. Mol Cell Endocrinol 317:25-30.

Sartori R, Milan G, Patron M, Mammucari C, Blaauw B, Abraham R, Sandri M. 2009. Smad2 and 3 transcription factors control muscle mass in adulthood. Am J Physiol Cell Physiol 296:C1248-57.

Schuelke M, Wagner KR, Stolz LE, Hubner C, Riebel T, Komen W, Braun T, Tobin JF, Lee SJ. 2004. Myostatin mutation associated with gross muscle hypertrophy in a child. N Engl J Med 350:2682-2688.

Schulz R, Vogel T, Dressel R, Krieglstein K. 2008. TGF-beta superfamily members, ActivinA and TGF-beta1, induce apoptosis in oligodendrocytes by different pathways. Cell Tissue Res 334:327-338.

Seale P, Rudnicki MA. 2000. A new look at the origin, function, and "stem-cell" status of muscle satellite cells. Dev Biol 218:115-24.

Seale P, Sabourin LA, Girgis-Gabardo A, Mansouri A, Gruss P, Rudnicki MA. 2000. Pax7 is required for the specification of myogenic satellite cells. Cell 102:777-786.

Sharma M, Kambadur R, Matthews KG, Somers WG, Devlin GP, Conaglen JV, Fowke PJ, Bass JJ. 1999. Myostatin, a transforming growth factor-beta super-family member, is expressed in heart muscle and is up-regulated in cardiomyocytes after infarct. J Cell Physiol 180:1-9.

Shelton GD, Engvall E. 2007. Gross muscle hypertrophy in whippet dogs is caused by a mutation in the myostatin gene. Neuromuscul Disord 17:721-722.

Shi Y, Massague J. 2003. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell 113:685-700.

Shyu KG, Ko WH, Yang WS, Wang BW, Kuan P. 2005. Insulin-like growth factor-1 mediates stretch-induced upregulation of myostatin expression in neonatal rat cardiomyocytes. Cardiovasc Res 68:405-414.

Sibley CP. In: Case RM, Waterhouse JM, editors. 1994. Human physiology: age, stress, and the environment. Oxford: Oxford University Press: p. 3-27.

Siriett V, Platt L, Salerno MS, Ling N, Kambadur R, Sharma M. 2006. Prolonged absence of myostatin reduces sarcopenia. J Cell Physiol 209:866-873.

Siriett V, Salerno MS, Berry C, Nicholas G, Bower R, Kambadur R, Sharma M. 2007. Antagonism of myostatin enhances muscle regeneration during sarcopenia. Mol. Ther. 15:1463–1470.

Siu PM. 2009. Muscle apoptotic response to denervation, disuse, and aging. Med. Sci. Sports Exerc. 41:1876–1886.

Smith DP, Fletcher DL. 1988. Chicken breast muscle fiber type and diameter as influenced by age and intramuscular location. Poult Sci 67:908-913.

Snow MH. 1977. The effects of aging on satellite cells in skeletal muscles of mice and rats. Cell and Tissue Research, 185:399-408.

Steven DH, London J. 1970. The foetus and its membranes. Med Biol Illus 20(4):218-219.

Stockdale FE, Nikovits W, Jr., Christ B. 2000. Molecular and cellular biology of avian somite development. Dev Dyn 219:304-321.

Stoikos CJ, Harrison CA, Salamonsen LA, Dimitriadis E. 2008. A distinct cohort of the TGF-β superfamily members expressed in human endometrium regulate decidualization. Hum Reprod 23: 1447-1456.

Summers PJ, Medrano JF. 1994. Morphometric analysis of skeletal-muscle growth in the high growth mouse. Grow Devel Aging 58:135-148.

Sundaresan NR, Saxena VK, Singh R, Jain P, Singh KP, Anish D, Singh N, Saxena M, Ahmed KA. 2008. Expression profile of myostatin mRNA during the embryonic organogenesis of domestic chicken (*Gallus gallus domesticus*). Res Vet Sci 85:86–91.

Swatland HJ, Kieffer NM. 1974. Fetal development of the double muscled condition in cattle. J Anim Sci 38:752-757.

Swatland HJ. 1975. Myofibre number and myofibrillar development in neonatal pigs. Zent Veterin A 22:756-763.

Takehara-Kasamatsu Y, Tsuchida K, Nakatani M, Murakami T, Kurisaki A, Hashimoto O, Ohuchi H, Kurose H, Mori K, Kagami S, Noji S, Sugino H. 2007. Characterization of follistatin-related gene as a negative regulatory factor for activin family members during mouse heart development. J Med Invest 54:276-288.

Taylor WE, Bhasin S, Artaza J, Byhower F, Azam M, Willard DH Jr, Kull FC Jr, Gonzalez-Cadavid N. 2001. Myostatin inhibits cell proliferation and protein synthesis in C2C12 muscle cells. Am J Physiol Endocrinol Metab 280:E221-E228.

Thayer M J, Tapscott S J, Davis R L, Wright W E, Lassar A B, Weintraub H. 1989. Positive autoregulation of the myogenic determination gene MyoD1. Cell 58:241-8.

Thies RS, Chen T, Davies MV, Tomkinson KN, Pearson AA, Shakey QA, Wolfman NM. 2001. GDF-8 propeptide binds to GDF-8 and antagonizes biological activity by inhibiting GDF-8 receptor binding. Growth Factors 18: 251-9.

Thomas M, Langley B, Berry C, Sharma M, Kirk S, Bass J, Kambadur R. 2000. Myostatin, a negative regulator of muscle growth, functions by inhibiting myoblast proliferation. J Biol Chem 275:40235-40243.

Toniolo L, Maccatrozzo L, Patruno M, Caliaro F, Mascarello F, Reggiani C. 2005. Expression of eight distinct MHC isoforms in bovine striated muscles: evidence for MHC-2B presence only in extraocular muscles. J Exp Biol 208:4243-4253.

Tsuchida K. 2004. Activins, myostatin and related TGF-beta family members as novel therapeutic targets for endocrine, metabolic and immune disorders. Curr Drug Targets Immune Endocr Metabol Disord 4:157-66.

Tsuchida K. 2008. Myostatin inhibition by a follistatin-derived peptide ameliorates the pathophysiology of muscular dystrophy model mice. Acta Myol 27:14-18.

Wagner KR, Liu X, Chang X, Allen RE. 2005. Muscle regeneration in the prolonged absence of myostatin. Proceedings of the National Academy of Sciences, 102:2519-2524.

Wagner KR, McPherron AC, Winik N, Lee SJ. 2002. Loss of myostatin attenuates severity of muscular dystrophy in mdx mice. Ann Neurol 52:832-836.

Wegner J, Albrecht E, Fiedler I, Teuscher F, Papstein HJ, Ender K. 2000. Growth-and breed-related. J Anim Sci 78: 1485- 496.

Wehling M, Cai B, Tidball JG. 2000. Modulation of myostatin expression during modified muscle use. FASEB J 14:103-110.

Weintraub H, Davis R, Tapscott S, Thayer M, Krause M, Benezra R, Blackwell T, Turner D, Rupp R, Hollenberg S. 1991. The myoD gene family: nodal point during specification of the muscle cell lineage. Science 251 (4995):761–6.

Weintraub H, Tapscott S J, Davis R L, Thayer M J, Adam M A, Lassar A B, Miller D. 1989. Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD. Proc. Natl. Acad. Sci. USA 86: 5434–5438.

Welle S, Burgess K, Mehta S. 2009. Stimulation of skeletal muscle myofibrillar protein synthesis, p70 S6 kinase phosphorylation, and ribosomal protein S6 phosphorylation by inhibition of myostatin in mature mice. Am. J. Physiol. 296:E567–E572.

Whittemore LA, Song K, Li X, et al. 2002. Inhibition of myostatin in adult mice increases skeletal muscle mass and strength. Biochem Biophys Res Commun 300:965-971.

Wolfman NM, McPherron AC, Pappano WN, Davies MV, Song K, Tomkinson KN, Wright JF, Zhao L, Sebald SM, Greenspan DS, Lee SJ. 2003. Activation of latent myostatin by the BMP-1/tolloid family of metalloproteinases. Proc Natl Acad Sci U S A 100:15842-15846.

Wong CL, Huang YY, Ho WK, Poon HK, Cheung PL, O WS, Chow PH. 2009. Growth-differentiation factor-8 (GDF-8) in the uterus: its identification and functional significance in the golden hamster. Reproduction Biology and Endocrinology. 7:134.

Wong ML, Medrano JF. 2005. Real-time PCR for mRNA quantitation. BioTechniques 39:75-85.

Xu Z, Ichikawa N, Kosaki K, Yamada Y, Sasaki T, Sakai LY, Kurosawa H, Hattori N, Arikawa-Hirasawa E. 2010. Perlecan deficiency causes muscle hypertrophy, a decrease in myostatin expression, and changes in muscle fiber composition. Matrix Biol 29:461-470.

Yablonka-Reuveni Z, Rivera AJ. 1994. Temporal expression of regulatory and structural muscle proteins during myogenesis of satellite cells on isolated adult rat fibers. Dev. Biol. 164:588-603.

Yagami-Hiromasa T, Sato T, Kurisaki T, Kamijo K, Nabeshima Y -I, Fujisawa-Sehara A. 1995. A metalloprotease-disintegrin participating in myoblast fusion. Nature 377: 652–656.

Yamanouchi K, Soeta C, Naito K, Tojo H. 2000. Expression of myostatin gene in regenerating skeletal muscle of the rat and its localization. Biochem Biophys Res Commun 270:510-516.

Yang J, Ratovitski T, Brady JP, Solomon MB, Wells KD, Wall RJ. 2001. Expression of Myostatin Pro Domain Results in Muscular Transgenic Mice. Mol Reprod Dev. 60:351-361.

Yang J, Zhao B. 2006. Postnatal expression of myostatin propeptide cDNA maintained high muscle growth and normal adipose tissue mass in transgenic mice fed high-fat diet. Molecular Reproduction and Development. 73:462-469.

Zachwieja JJ, Smith SR, Sinha-Hikim I, Gonzalez-Cadavid N, Bhasin S. 1999. Plasma myostatin-immunoreactive protein is increased after prolonged bed rest with low-dose T3 administration. J Gravit Physiol 6: 11-15.

Zammit PS, Golding JP, Nagata Y, Hudon V, Partridge TA, Beauchamp JR. 2004. Muscle satellite cells adopt divergent fates: a mechanism for self-renewal. J. Cell Biol. 166:347-357.

Zammit PS, Relaix F, Nagata Y, Ruiz AP, Collins CA, Partridge TA, Beauchamp JR. 2006. Pax7 and myogenic progression in skeletal muscle satellite cells. J. Cell Sci. 119:1824-1832.

Zhang C, Tan CK, McFarlane C, Sharma M, Tan NS, Kambadur R. 2012. Myostatin-null mice exhibit delayed skin wound healing through the blockade of transforming growth factor-β signaling by decorin. Am Physiol Cell Physiol 302:C1213-C1225.

Zhang W, Behringer RR, Olson EN. 1995. Inactivation of the myogenic bHLH gene MRF4 results in up-regulation of myogenin and rib anomalies. Genes and Development 9:1388-1399.

Zhao B, Li E J, Wall R J, Yang J. 2009. Coordinated patterns of gene expressions for adult muscle build-up in transgenic mice expressing myostatin propeptide. BMC Genomics10:305.

Zhao B, Wall RJ, Yang J. 2005. Transgenic expression of myostatin propeptide prevents diet-induced obesity and insulin resistance. Biochem Biophys Res Commun 337:248-255.

Zhou J, Bondy C A. 1993. Placental glucose transporter gene expression and metabolism in the rat. The Journal of Clinical Investigation 91:845-852.

Zhu MJ, Ford SP, Nathanielsz PW, Du M. 2004. Effect of maternal nutrient restriction in sheep on the development of fetal skeletal muscle. Biol Reprod 71:1968-73.

Zimmers TA, Davies MV, Koniaris LG, Haynes P, Esquela AF, et al. 2002. Induction of cachexia in mice by systemically administered myostatin. Science 296:1486–1488.

4.4 Appendices

4.4.1 Appendix A: DNA Extraction from Animal Tissue

1. Proteinase K digestion
    a. Proteinase K: 10 mg /ml in 10 mM Tis (PH 8.0).
    b. Buffer (50 ml).
        i. 2.5 ml 10% SDS

ii. 2.5 ml 1M Tris,PH 8.0

iii. 10.0 ml 0.5 M EDTA

iv. 35 ml,dd H2O

Add 500 µl of buffer and 35 µl Proteinase K to each tube with mouse tail tissue (50 mg-100 mg) in 1.5 ml micro-centrifuge tube.

Over-night digestion at 50 °C.

2. Phenol and chloroform extraction

Open all the tubes and add 250 µl phenol and 250 µl chloroform, close tubes and vetex for 10 seconds.

3- Spin at 14,000 rpm for 5 minutes

While spinning, label 1.5 ml tube with animal ID numbers.

4-Carefully transfer the top phase (aqueous layer) without disturbing the lower protein layer to the newly labeled tubes. Normally, the volume is 450-500 µl.

5- Add 500 µl of chloroform to each tube, close the tube and vetax for 10 seconds.

6- Close all tubes and place them in centrifuge with the lid hinge pointing toward the center of the centrifuge, spin at highest for 2 minutes.

-While spinning, label 1.5 ml tube with animal ID numbers.

7- Transfer 400-450 µl of the top phase to the new tubes, changing pipette tips for each tube and make sure that the tube numbers are correct.

8- Add 45 µl of 3.0 M sodium acetate, PH 7.8 to each tube (10% of the volume for precipitation of DNA).

9- Add 1 ml of 100% Ethanol to each tube, close it and votex for 10 seconds; centrifuge the tubes at top speed for 10 minutes, with hinge facing the outside of the centrifuge.

10- Carefully remove the supernatant and dry the DNA pellets until they are clear. not white.

11- Add 1 ml 70% Ethanol to each tube, for washing the pellet, centrifuge for 10 mints, and dry DNA.

12. Add 50 µl ddH2O and put in water bath at 40°C for 1-2 hours.

13. Quantify DAN in a spectrophotometer. Write DNA concentration on the tube (µg/ µl).

4.4.2 Appendix B: qReal-Time PCR Protocol

1. Total RNA was isolated from tissues with TRIzol Reagent (Invitrogen) following manufacturer's instruction manual.

2. Genomic DNA residual in isolated total RNA was digested using Deoxyribonuclease 1, Amplification Grade (Invitrogen).

a) 5 µg total RNA + 1 µl 10x DNase 1 reaction buffer + 1 U DNase 1 + DEPC-treated water to 10 µl. (This step was done on ice)
b) The tube was incubated for 15 minutes at room temperature.
c) 1µl of 25 Mm EDTA was added to inactivate DNase 1. The reaction was heated for 10 minutes at 65°C.

3. First-Strand cDNA Synthesis Using SuperScript^{TM} III RT (Invitrogen)
A 20 µl reaction volume can be used for 1 ng-5 µg of total RNA or 1-500 ng of mRNA.

a) The following components were added to the above tube containing genomic DNA-free total RNA (1-1.5 µg):
1 µl Oligo (dT) 12-18 (500 µg/ml)
1 µl dNTP Mix (10 mM each)
b) The mixture was heated to 65°C for 5 min and was then quickly chilled on ice for 2 minutes.
c) The contents in the tube were collected by brief centrifugation. 4 µl of 5X First-Strand Buffer, 1 µl of 0.1 MDTT and 1 µl of RNaseOUT (40U) were added.
d) 1 µl (200 units) of SuperScript^{TM} III RT was added and mixed by pipetting gently up and down.
e) The reaction was incubated at 50 °C for 30 – 60 minutes.
f) The reaction was inactivated by heating at 70 °C for 15 minutes.
g) 50 µl of ddH_2O was added to each tube and mixed to make the concentration of cDNA to equal to 10 ng/µl. 5µl of first strand cDNA (approximately containing 50 ng of total RNA) was taken as template for the 50 µl reaction volume/ tube real-time PCR.

4. Real-time PCR:

a) The following components were added to each well of 96-well plate:

5 µl of cDNA template (approximately containing 50 ng of total RNA)
2.5 µl of Forward primer (1 pmol/ µl), with final concentration of 5 nm
2.5 µl of Reverse primer (1 pmol/ µl), with final concentration of 5 nm
25 µl of Power SYBR green PCR master mix (2x, without UNG enzyme)
15 µl of distilledH_2O

b) The plate was sealed with optical membrane, and vortexed for 10 seconds. The plate was centrifuged for 1 minute at 2000g.
c) An "sds" plate document was created and saved in the computer that controls the real-time PCR machine to store the data obtained during real-time PCR.
d) The real-time PCR was performed. The thermal cycling parameters were set as followed:
   Optimal annealing temperatures for the primers used was determined to be 60 °C, cDNA with thermal cycle 94°C 4 Minutes and 42 cycles of 94 °C for 40 Seconds, 60 °C for 30 Seconds, and 72 °C for 30 Seconds were run.

5. At the end of the reaction, dissociation curve was created to verify the identity and specificity of the amplification products.
6. All myostatin mRNA values were normalized to an endogenous reference gene (β-actin) and relative to untreated control through the formula $2^{-\Delta\Delta Ct}$.

4.4.3 Appendix C: The raw data of table 2.1

Measurements at day 10 of pregnancy for Wild-Type group:

Meas / ID	**155**	**706**	**703**	**208**	**251**	**254**	**250**
Live Wt. (g)	38.3	32.6	37.8	22.1	25.6	27.3	30.59
Littersize	10	6	5	8	6	7	7
Total Fetal Wt. (g)	1	0.6	1.6	0.5	1.6	2.1	1.17
Individual fetal wt. (g)	0.1	0.1	0.32	0.06	0.26	0.3	0.16

Uterine wt. (g)	4.2	2.5	4.1	1.6	3.7	4.8	3.48
Uterine length (cm)	7.2	6	7.5	5.6	7	9	7
Uterine width (cm)	1.3	1	1.5	1	1	1	1.13
Placenta. Wt. (g)	1.4	0.6	0.7	0.8	1.5	1.6	1.1
Placenta ratio	1.4	1	0.44	1.6	0.94	0.76	1

Measurements at day 10 of pregnancy for ($MSTN^{TG/+}$) transgenic group:

Meas \ ID	**178**	**195**	**217**	**216**	**253**	**252**	**255**
Live Wt. (g)	36.8	43.5	32.1	37.7	31.6	35.44	37.24
Littersize	7	10	6	9	6	9	7
Total Fetal Wt. (g)	2.66	4.3	2.4	3.24	2.04	3.33	2.66
Individual fetal wt. (g)	0.38	0.43	0.4	0.36	0.34	0.37	0.38
Uterine wt. (g)	6.5	6.8	4.2	6.3	5.26	5.9	6.2
Uterine length (cm)	7.9	8.5	6.8	8.5	7.8	10.45	9.6
Uterine width (cm)	1.2	1.5	1.4	1.2	1	1.2	1.3
Placenta. Wt. (g)	0.8	1.8	1.2	1.8	1.6	1.8	1.5
Placenta ratio	0.3	0.42	0.5	0.55	0.78	0.54	0.56

Measurements at day 10 of pregnancy for ($MSTN^{TG/TG}$) transgenic group:

Meas \ ID	**White**	**A**	**256**	**257**	**258**	**259**	**260**
Live Wt. (g)	40.3	45.3	42	43.8	42.8	43.2	42.24
Littersize	7	8	8	7	8	6	9
Total Fetal	1.8	6.5	4.3	4	5.3	3	4.4

Wt. (g)							
Individual fetal wt. (g)	0.26	0.81	0.54	0.57	0.66	0.5	0.49
Uterine wt. (g)	4.4	10.2	9	8	6	7.3	6.2
Uterine length (cm)	7	11.5	11.2	9.4	10	8.4	7.2
Uterine width (cm)	1.2	1.5	1.4	1.5	1.3	1.2	1.35
Placenta. Wt. (g)	1.7	2.3	2.4	1.6	1.2	2.8	2
Placenta ratio	0.94	0.35	0.56	0.4	0.23	0.93	0.45

4.4.4 Appendix D: The raw data of table 2.2:

Measurements at day 16 of pregnancy for Wild-Type group:

Meas \ ID	**194**	**218**	**158**	**702**	**261**	**262**	**373**
Live Wt. (g)	41.8	39.6	44.1	43.7	36.9	31.8	39.65
Littersize	7	10	10	7	9	7	8
Total Fetal Wt. (g)	5.6	7.5	8.8	4.6	8.2	6.37	7.84

Individual fetal wt. (g)	0.8	0.75	0.88	0.66	0.91	0.91	0.98
Uterine wt. (g)	11	11.2	13.1	7.4	10.7	9.8	12.2
Uterine length (cm)	8.8	9.8	9	9.6	8.6	10.4	10
Uterine width (cm)	1.8	1.5	1.6	1.3	1.4	1.4	1.2
Placenta. Wt. (g)	1.95	2.2	1.8	1.6	1.5	1.4	1.7
Placenta ratio	0.35	0.29	0.2	0.35	0.18	0.22	0.22

Measurements at day 16 of pregnancy for ($MSTN^{TG/+}$) transgenic group:

Meas \ ID	**232**	**233**	**154**	**220**	**169**	**170**	**181**
Live Wt. (g)	45.4	44.6	41.2	33.5	44.8	44.2	48.2
Littersize	7	5	10	7	7	8	10
Total Fetal Wt. (g)	7.8	7.4	7.4	8.3	8.6	9	9.02
Individual fetal wt. (g)	1.11	1.48	0.74	1.19	1.23	1.13	0.902
Uterine wt. (g)	12.3	9.48	10.7	12.7	10.19	12.2	12.15
Uterine length (cm)	9.2	10.8	10.6	9.7	10.4	10.5	12.3
Uterine width (cm)	1.6	1.4	1.8	1.4	1.8	1.4	1.8
Placenta. Wt. (g)	1.3	1.4	2	1.5	1.8	2.4	2.35
Placenta ratio	0.16	0.19	0.27	0.18	0.2	0.27	0.26

Measurements at day 16 of pregnancy for ($MSTN^{TG/TG}$) transgenic group:

Meas \ ID	**364**	**365**	**367**	**369**	**370**	**371**	**372**
Live Wt. (g)	46	46	46	47	46	47	47.5
Littersize	10	9	9	10	10	10	9
Total Fetal	15.7	14.4	10.44	14.4	15.2	12	12.6

Wt. (g)							
Individual fetal wt. (g)	1.57	1.6	1.16	1.44	1.52	1.2	1.4
Uterine wt. (g)	17.6	16.6	12.2	16.2	16	14	14
Uterine length (cm)	12.5	10.5	14.4	13.8	15	14.6	13.6
Uterine width (cm)	1.8	1.4	1.6	1.8	1.8	1.4	1.4
Placenta. Wt. (g)	2.4	1.6	2.4	2.8	2.6	2.8	2.4
Placenta ratio	0.15	0.11	0.23	0.19	0.17	0.23	0.19

4.4.5 Appendix E: The raw data of qRT-PCR for figures 2.4, 2.5, and 2.6:

Myostatin gene expression Ct value and β-actin Ct value:
A1-A9, Myostatin gene in wild type group at day 10 of pregnancy
A10-A12, β-actin gene in wild type group at day 10 of pregnancy
B1-B9, Myostatin gene in TG ($MSTN^{TG/+}$) group at day 10 of pregnancy
B10-B12, β-actin gene in TG ($MSTN^{TG/+}$) group at day 10 of pregnancy
C1-C9, Myostatin gene in TG ($MSTN^{TG/TG}$) group at day 10 of pregnancy
C10-C12, β-actin gene in TG ($MSTN^{TG/TG}$) group at day 10 of pregnancy

D1-D9, Myostatin gene in wild type group at day 16 of pregnancy
D10-D12, β-actin gene in wild type group at day 16 of pregnancy
E1-E9, Myostatin gene in TG ($MSTN^{TG/+}$) group at day 16 of pregnancy
E10-E12, β-actin gene in TG ($MSTN^{TG/+}$) group at day 16 of pregnancy
F1-F9, Myostatin gene in TG ($MSTN^{TG/TG}$) group at day 16 of pregnancy
F10-F12, β-actin gene in TG ($MSTN^{TG/TG}$) group at day 16 of pregnancy

Gene	Ct	Gene	Ct
A1	35.54	D1	37.93
A2	34.99	D2	41.23
A3	35.05	D3	38.25
A4	39.98	D4	37.37
A5	38.75	D5	38.84
A6	40.47	D6	37.1
A7	36.41	D7	38.45
A8	36.49	D8	38
A9	36.38	D9	38.9
A10	18.1	D10	19.19
A11	17.59	D11	19.27
A12	17.87	D12	19.43
B1	33.68	E1	39.66
B2	34.05	E2	38.26
B3	33.71	E3	39.83
B4	35.29	E4	35.2
B5	35.2	E5	35.43
B6	35.38	E6	34.85
B7	35.91	E7	37.2
B8	37.13	E8	37
B9	37.27	E9	37.4
B10	19.92	E10	20.76
B11	19.81	E11	20.41
B12	19.54	E12	20.36
C1	34.32	F1	34.63
C2	34.8	F2	34.73
C3	35.42	F3	34
C4	34.14	F4	34.53
C5	34	F5	34.2
C6	34.15	F6	35.16
C7	34.56	F7	34
C8	34	F8	34.8
C9	35	F9	34.6
C10	18.38	F10	18.65
C11	18.11	F11	17.35
C12	18.11	F12	17.33

Calculation:

ΔCt = Ct Target Gene (TG) – Ct Reference Gene (β-actin)

ΔCt = Ct Calibrated Gene (WT) – Ct Reference Gene (β-actin)

$\Delta\Delta Ct = \Delta Ct$ Target Gene (TG) - ΔCt Calibration Gene (WT)

Fold Change = $2^{-\Delta\Delta Ct}$

4.4.6 Appendix F: The raw data of Table 3.2:

Genotype	Age	Rep.	Id	LW	Soleus	Plantares	Gastro	Carcass	White	Brown
TG	1 month	1	362	24.63	7	19	137.5	9.8	194	68
TG	1 month	2	363	22.96	6	17	115.5	9	152	116
TG	1 month	3	366	21.28	5	16.5	124.5	7.9	181	110
TG	1 month	4	367	24.15	7	25	138	9.57	189	90
TG	1 month	5	355	22.7	7	16.5	131	9.4	185	193
TG	1 month	6	356	21.24	5	14	124	8.042	139	99
WT	1 month	1	370	22.18	5	10	99	7.3	130	75
WT	1 month	2	377	21.19	4	10	98	7	117	72
WT	1 month	3	327	19.71	2.5	10	84.5	6.62	112	78
WT	1 month	4	368	20.27	5	10	85	6.8	94	80
WT	1 month	5	374	19.06	5	8.5	81	6.4	109	87
WT	1 month	6	351	20.11	4.5	10.5	95.5	6.8	200	98
WT	1 month	7	848	18.92	4	10	81.5	6.4	192	82
WT	1 month	8	353	20.91	4.5	16.5	111.5	7.4	217	108
WT	1	9	925	21.26	5	11.5	99.5	7.1	199	113

	month									
TG	6 month	1	702	34	9.5	39.5	228.5	16.2	304	127
TG	6 month	2	712	38.4	8	30	248	18.97	147	146
TG	6 month	3	713	35.4	9.5	28.5	229	17.15	106	140
TG	6 month	4	714	37.8	11	40	253	18.55	280	121
TG	6 month	5	715	33.2	8.5	31.5	216.5	17.28	135	116
TG	6 month	6	706	33.9	9	26.5	242	16.04	131	81.7
TG	6 month	7	774	34.4	5	30	196	15.8	168	141
WT	6 month	1	701	31.8	8	20	170	13.34	375	153
WT	6 month	2	705	30.3	7.5	20	170.5	12.36	231	108
WT	6 month	3	707	27	13	19	150.5	11.19	438	104
WT	6 month	4	708	29.3	4.5	20.5	167.5	11.91	320	121
WT	6 month	5	768	27.1	5.5	19	183	11.54	323	107
WT	6 month	6	773	28	6.5	22.5	135	11.87	391	123
WT	6 month	7	775	29.3	6.5	26.5	192	13.62	210	98
TG	12	1	263	37.8	9.5	40	254	17.88	228	140

	month									
TG	12 month	2	256	37.9	7.5	24.5	242	18.38	412	141
TG	12 month	3	264	36.3	19	29.5	258.5	17.56	313	182
TG	12 month	4	269	41.7	12.5	29	232.5	19.27	1777	196
WT	12 month	1	202	39.3	8.5	16	162.5	13.9265	1130	156
WT	12 month	2	234	33.1	9	14.5	136	12.9025	713	193
WT	12 month	3	259	29.8	8	23.5	156	11.117	815	142
WT	12 month	4	265	33.5	8.5	18	138.5	12.161	869	186
WT	12 month	5	255	36.4	12	32.5	257	17.1445	636	153
WT	12 month	6	275	28.8	8.5	18.5	161.5	10.9155	381	124
WT	12 month	7	274	29.1	8.5	16.5	151.5	11.1005	449	124
WT	12 month	8	257	39.7	9.5	22	168	14.9875	1478	258
TG	18 month	1	600	34.9	8.6	18.5	117.5	8.6335	10	41.5
TG	18 month	2	200	38.8	10	26	205	17.635	343	156
TG	18 month	3	174	35.7	8.65	24.5	185	15.881	260	153
TG	18	4	176	36.2	7.85	32	195	17.0715	139	174

	month									
TG	18 month	5	189	34.5	7.68	21.5	150	14.163	384	184
TG	18 month	6	191	27.1	10	38	171	10.811	380	134
TG	18 month	7	163	31.3	9.5	24	181	12.9025	145	149
TG	18 month	8	161	36.7	10	27	186.5	16.0805	143	131
TG	18 month	9	160	41.2	9.85	21	192	17.8465	137	135
WT	18 month	1	167	31.6	7.5	17	128.5	9.8265	495	146
WT	18 month	2	199	48	6.5	18.5	131.5	12.6865	2254	196
WT	18 month	3	162	40	8	18	134.5	11.5805	972	160
WT	18 month	4	165	43.8	6.5	18	213	13.629	797	217

4.4.7 Appendix G: The raw data of Ct value for Myostatin gene in qRT-PCR:

Gene	Ct	Gene	Ct	Gene	Ct	Gene	Ct	Gene	Ct	Gene	Ct
A1	33.16	D1	28.84	G1	32.61	J1	30.95	M1	32.31	P1	29.57
A2	33.14	D2	28.3	G2	31.89	J2	29.96	M2	31.16	P2	29.29
A3	32.26	D3	29	G3	32.31	J3	30.78	M3	30.53	P3	29.13
A4	34.7	D4	25.05	G4	30.44	J4	28.55	M4	33.76	P4	32.26
A5	34.27	D5	24.4	G5	30.18	J5	29.12	M5	34.05	P5	32.84
A6	33.74	D6	23.67	G6	30.27	J6	28.08	M6	34.21	P6	32.41
A7	33.64	D7	26.06	G7	29.96	J7	30.69	M7	33.38	P7	31.02
A8	32.05	D8	24.99	G8	29.32	J8	30.8	M8	32.93	P8	31.06
A9	31.99	D9	26.43	G9	29.45	J9	31.32	M9	32.93	P9	31.23
B1	28.22	E1	29.53	H1	30.68	K1	32.04	N1	31.22	-	-
B2	27.66	E2	29.52	H2	31.4	K2	30.75	N2	30.12	-	-
B3	28.34	E3	29.33	H3	30.21	K3	30.64	N3	30.35	-	-
B4	28.67	E4	31.16	H4	28.38	K4	38.37	N4	30.5	-	-
B5	28.77	E5	30.99	H5	28.67	K5	38.4	N5	30.4	-	-
B6	28.45	E6	31.1	H6	28.79	K6	39.02	N6	30.59	-	-
B7	28.42	E7	27.96	H7	27.62	K7	30.82	N7	32.72	-	-
B8	28.38	E8	27.51	H8	27.52	K8	30.85	N8	31.97	-	-
B9	28.48	E9	27.53	H9	27.57	K9	30.58	N9	31.6	-	-
C1	33.45	F1	28.17	I1	32.94	L1	29.38	O1	31.71	-	-
C2	34.04	F2	28.12	I2	32.15	L2	29.1	O2	32.17	-	-
C3	33.33	F3	27.94	I3	32.25	L3	29.23	O3	31.22	-	-
C4	28.11	F4	28.46	I4	31.12	L4	31.81	O4	35.25	-	-
C5	27.58	F5	28.69	I5	31.09	L5	32.13	O5	35.61	-	-
C6	27.77	F6	28.49	I6	31.09	L6	31.99	O6	36.63	-	-
C7	29.84	F7	26.81	I7	34.62	L7	29.29	O7	33.07	-	-
C8	31.04	F8	27.68	I8	34.69	L8	29.02	O8	32.79	-	-
C9	29.58	F9	26.32	I9	35.16	L9	29.24	O9	33.49	-	-

A1-A9: Myostatin gene Ct value 1 month old WT male.
B1-B9: β-actin gene Ct value 1 month old WT male.
C1-C9: Myostatin gene Ct value 1 month old TG male.
D1-D9: β-actin gene Ct value 1 month old TG male.
E1-E9: Myostatin gene Ct value 6 month old WT male.
F1-F9: β-actin gene Ct value 6 month old WT male.
G1-G9: Myostatin gene Ct value 6 month old TG male.
H1-H9: β-actin gene Ct value 6 month old TG male.
I1-I9: Myostatin gene Ct value 12 month old WT male.
J1-J9: β-actin gene Ct value 12 month old WT male.
K1-K9: Myostatin gene Ct value 12 month old TG male.
L1-L9: β-actin gene Ct value 12 month old TG male.
M1-M9: Myostatin gene Ct value 18 month old WT male.
N1-N9: β-actin gene Ct value 18 month old WT male.
O1-O9: Myostatin gene Ct value 18 month old TG male.
P1-P9: β-actin gene Ct value 18 month old TG male.

4.4.8 Appendix H: The raw data of Ct value for MyoD gene in qRT-PCR:

Gene	Ct	Gene	Ct	Gene	Ct	Gene	Ct	Gene	Ct	Gene	Ct
A1	34.2	D1	28.07	G1	34.9	J1	28.75	M1	32.64	P1	28.77
A2	33.1	D2	27.76	G2	33.55	J2	28.09	M2	34.26	P2	28.57
A3	33.07	D3	27.99	G3	33.06	J3	28.91	M3	32.77	P3	28.2
A4	34.08	D4	24.28	G4	32.97	J4	27.36	M4	35.2	P4	31.28
A5	34.08	D5	24.73	G5	33.85	J5	27.77	M5	34.4	P5	31.02
A6	34.39	D6	25.88	G6	33.35	J6	27.41	M6	33.2	P6	31.21
A7	33.02	D7	26.61	G7	32.49	J7	30.03	M7	37.28	P7	30.11
A8	32.96	D8	26.2	G8	32.08	J8	30.75	M8	0	P8	30.29
A9	33.16	D9	0	G9	32.49	J9	30.03	M9	37.87	P9	30.05
B1	27.68	E1	31.23	H1	30.03	K1	34.87	N1	29.03	-	-
B2	27.08	E2	31.09	H2	29.7	K2	34.9	N2	28.24	-	-
B3	27.23	E3	30.71	H3	29.97	K3	35.23	N3	28.41	-	-
B4	27.99	E4	32.84	H4	28.28	K4	33.8	N4	28.77	-	-
B5	28.16	E5	33.88	H5	28.39	K5	34.2	N5	28.89	-	-
B6	28	E6	33.69	H6	28.2	K6	35.4	N6	29.01	-	-
B7	27.05	E7	29.74	H7	26.99	K7	34.4	N7	30.16	-	-
B8	27.75	E8	31.63	H8	27.07	K8	34.78	N8	30.74	-	-
B9	27.01	E9	29.95	H9	27.09	K9	34.5	N9	30.02	-	-
C1	35.45	F1	27.03	I1	32.27	L1	28.11	O1	39.62	-	-
C2	35.9	F2	27.22	I2	32.05	L2	27.86	O2	38.08	-	-
C3	34.82	F3	27.08	I3	31.8	L3	27.93	O3	0	-	-
C4	28.74	F4	27.7	I4	34.09	L4	30.19	O4	37.2	-	-
C5	29.26	F5	27.6	I5	34.61	L5	30.21	O5	38.1	-	-
C6	30.11	F6	28.03	I6	34.06	L6	30.34	O6	36	-	-
C7	30.66	F7	25.25	I7	36.74	L7	28.07	O7	37.59	-	-
C8	31	F8	24.94	I8	35.81	L8	28.6	O8	35.64	-	-
C9	0	F9	26.12	I9	38.05	L9	28.27	O9	37.22	-	-

A1-A9: MyoD gene Ct value 1 month old WT male.
B1-B9: β-actin gene Ct value 1 month old WT male.
C1-C9: MyoD gene Ct value 1 month old TG male.
D1-D9: β-actin gene Ct value 1 month old TG male.
E1-E9: MyoD gene Ct value 6 month old WT male.
F1-F9: β-actin gene Ct value 6 month old WT male.
G1-G9: MyoD gene Ct value 6 month old TG male.
H1-H9: β-actin gene Ct value 6 month old TG male.
I1-I9: MyoD gene Ct value 12 month old WT male.
J1-J9: β-actin gene Ct value 12 month old WT male.
K1-K9: MyoD gene Ct value 12 month old TG male.
L1-L9: β-actin gene Ct value 12 month old TG male.
M1-M9: MyoD gene Ct value 18 month old WT male.
N1-N9: β-actin gene Ct value 18 month old WT male.
O1-O9: MyoD gene Ct value 18 month old TG male.
P1-P9: β-actin gene Ct value 18 month old TG male.

4.4.9 Appendix I: The raw data of Ct value for Myf5 gene in qRT-PCR:

Gene	Ct	Gene	Ct	Gene	Ct	Gene	Ct	Gene	Ct	Gene	Ct
A1	30.98	D1	23	G1	38.55	J1	27.74	M1	38.16	P1	26.21
A2	30.72	D2	23.37	G2	34.77	J2	27.31	M2	37.8	P2	31.15
A3	30.42	D3	22.6	G3	40.75	J3	26.69	M3	37.98	P3	27.2
A4	32.01	D4	22.42	G4	0	J4	0	M4	36.8	P4	28.4
A5	31.56	D5	23.1	G5	39.15	J5	37.23	M5	36.9	P5	26.3
A6	31.46	D6	22.26	G6	34.67	J6	37.09	M6	37.4	P6	27.8
A7	31.48	D7	21.67	G7	34.98	J7	26.2	M7	38.2	P7	30.86
A8	31.09	D8	22.37	G8	0	J8	27.3	M8	36.8	P8	28.71
A9	31.01	D9	22.27	G9	35.39	J9	28.1	M9	37.7	P9	31.75
B1	22.6	E1	32	H1	0	K1	37.07	N1	37.07	-	-
B2	22.65	E2	31.18	H2	26.76	K2	36.6	N2	26.39	-	-
B3	22.28	E3	31.2	H3	26.76	K3	37.2	N3	26.65	-	-
B4	22.96	E4	31.8	H4	28.85	K4	37.6	N4	27.88	-	-
B5	23	E5	31.6	H5	28.82	K5	36.8	N5	32.13	-	-
B6	23.53	E6	31.62	H6	28.78	K6	37.1	N6	32.79	-	-
B7	23.67	E7	34.05	H7	26.11	K7	36.7	N7	35	-	-
B8	22.96	E8	31.19	H8	26.16	K8	37.4	N8	0	-	-
B9	22.91	E9	31.38	H9	27.88	K9	38.2	N9	36.11	-	-
C1	32.41	F1	28.4	I1	38.2	L1	37.8	O1	36.08	-	-
C2	32.06	F2	28.26	I2	38.1	L2	38.22	O2	37.94	-	-
C3	31.7	F3	26.05	I3	37.9	L3	39.01	O3	38.2	-	-
C4	31.26	F4	27.24	I4	37.2	L4	33.42	O4	39.1	-	-
C5	30.77	F5	26.74	I5	37.1	L5	0	O5	39.4	-	-
C6	31.52	F6	27.37	I6	37.3	L6	30.85	O6	41.64	-	-
C7	29.15	F7	23.92	I7	38.1	L7	27.28	O7	39.2	-	-
C8	28.06	F8	24.11	I8	37.9	L8	27.33	O8	38.2	-	-
C9	0	F9	23.12	I9	36.8	L9	32.85	O9	37.98	-	-

A1-A9: Myf5 gene Ct value 1 month old WT male.
B1-B9: β-actin gene Ct value 1 month old WT male.
C1-C9: Myf5 gene Ct value 1 month old TG male.
D1-D9: β-actin gene Ct value 1 month old TG male.
E1-E9: Myf5 gene Ct value 6 month old WT male.
F1-F9: β-actin gene Ct value 6 month old WT male.
G1-G9: Myf5 gene Ct value 6 month old TG male.
H1-H9: β-actin gene Ct value 6 month old TG male.
I1-I9: Myf5 gene Ct value 12 month old WT male.
J1-J9: β-actin gene Ct value 12 month old WT male.
K1-K9: Myf5 gene Ct value 12 month old TG male.
L1-L9: β-actin gene Ct value 12 month old TG male.
M1-M9: Myf5 gene Ct value 18 month old WT male.
N1-N9: β-actin gene Ct value 18 month old WT male.
O1-O9: Myf5 gene Ct value 18 month old TG male.
P1-P9: β-actin gene Ct value 18 month old TG male.

4.4.10 Appendix J: The raw data of Ct value for Pax7 gene in qRT-PCR:

Gene	Ct	Gene	Ct	Gene	Ct	Gene	Ct	Gene	Ct	Gene	Ct
A1	0	D1	28.16	G1	37.32	J1	34.89	M1	36.94	P1	35.39
A2	37.17	D2	27.94	G2	37.28	J2	34.28	M2	37.62	P2	35.65
A3	34.83	D3	28.01	G3	37.15	J3	34.24	M3	37.86	P3	37
A4	34.22	D4	25.14	G4	36.6	J4	32.99	M4	38	P4	36.51
A5	37.45	D5	25.2	G5	36.4	J5	33.18	M5	37.8	P5	36.47
A6	36.42	D6	25.21	G6	36.5	J6	32.72	M6	37.4	P6	36.42
A7	0	D7	26.14	G7	35.7	J7	35.23	M7	36.8	P7	35.52
A8	37.93	D8	26.33	G8	37.43	J8	35.53	M8	37.2	P8	36.18
A9	36.24	D9	26.98	G9	35.67	J9	36.19	M9	37.6	P9	36.66
B1	27.24	E1	36.01	H1	29.1	K1	37.4	N1	34.51	-	-
B2	26.91	E2	35.4	H2	29.21	K2	38.48	N2	34.09	-	-
B3	27.11	E3	37.58	H3	28.79	K3	37.8	N3	34.97	-	-
B4	27.05	E4	36.8	H4	27.19	K4	36.4	N4	34.47	-	-
B5	27.34	E5	0	H5	27.59	K5	36.8	N5	34.27	-	-
B6	27.39	E6	37.09	H6	27.37	K6	37.2	N6	34.42	-	-
B7	26.95	E7	36.07	H7	26.57	K7	37.38	N7	35.39	-	-
B8	27.27	E8	35.47	H8	26.73	K8	37.8	N8	36.33	-	-
B9	27.04	E9	36.48	H9	26.92	K9	36.8	N9	36.63	-	-
C1	0	F1	26.75	I1	38.4	L1	37.38	O1	36.2	-	-
C2	37.36	F2	27.15	I2	37.79	L2	33.88	O2	37.6	-	-
C3	36.09	F3	27.04	I3	37.2	L3	33.84	O3	36.38	-	-
C4	36.27	F4	27.6	I4	37.1	L4	37.14	O4	37.99	-	-
C5	37.67	F5	27.86	I5	36.8	L5	36.5	O5	37.3	-	-
C6	37.21	F6	28.08	I6	35.84	L6	36.17	O6	37.2	-	-
C7	34.75	F7	25.7	I7	37.4	L7	33.84	O7	36.4	-	-
C8	35.75	F8	25.68	I8	37.6	L8	34	O8	36.54	-	-
C9	35.03	F9	26.04	I9	37.45	L9	34.62	O9	36.2	-	-

A1-A9: Pax7 gene Ct value 1 month old WT male.
B1-B9: β-actin gene Ct value 1 month old WT male.
C1-C9: Pax7 gene Ct value 1 month old TG male.
D1-D9: β-actin gene Ct value 1 month old TG male.
E1-E9: Pax7 gene Ct value 6 month old WT male.
F1-F9: β-actin gene Ct value 6 month old WT male.
G1-G9: Pax7 gene Ct value 6 month old TG male.
H1-H9: β-actin gene Ct value 6 month old TG male.
I1-I9: Pax7 gene Ct value 12 month old WT male.
J1-J9: β-actin gene Ct value 12 month old WT male.
K1-K9: Pax7 gene Ct value 12 month old TG male.
L1-L9: β-actin gene Ct value 12 month old TG male.
M1-M9: Pax7 gene Ct value 18 month old WT male.
N1-N9: β-actin gene Ct value 18 month old WT male.
O1-O9: Pax7 gene Ct value 18 month old TG male.
P1-P9: β-actin gene Ct value 18 month old TG male.