Investigation into Developmental Stages of Chicken and Quail Blood to Determine Alternative Splicing Isoforms of Myostatin

Jessica Wheeler
Research Thesis
Advisor: Dr. Kichoon Lee
Department of Animal Sciences
The Ohio State University
2013

INTRODUCTION

Very little is known or understood about avian muscle development especially during neonatal growth stages. Understanding this stage of development leads to the advancement of muscle growth and an influence on fat deposition. This ultimately leads to increased efficiency in animal production. In future years, the world faces the momentous problem of a 50% global population increase to at least 9 billion people. The present equipment, available food, and land to raise livestock will not satisfy the future food demand of 2050 (Khan et al., 2009). Enhancing meat production efficiency will influence the food demand by creating new food availability solutions. Understanding muscle development and how myostatin influences cellular growth significantly benefits poultry producers as the slight increases in muscle mass have dramatic effects on the gain-to-feed ratio and the efficiency of producing the animals. Comprehension of muscle development opens up other areas of study including progress in reversing muscle loss in patients with muscular atrophy, muscle wasting diseases, and understanding evolutionary processes. Investigating the molecular process of myostatin leads to understanding minor changes in gene structure, function, and differences between species (Rodgers et al., 2008).

Myostatin, or growth and differentiation factor-8 (GDF-8), negatively regulates skeletal muscle growth. As a member of the transforming growth factor-beta (TGF-β) super family of growth factors, myostatin propeptide is synthesized as a precursor protein that is cleaved by furin proteases. The furin proteases separate the N-terminal propeptide from the bioactive C-terminal domain, which forms a disulfide-linked dimer (Rodgers et al., 2008). The dimer then binds to receptors and inhibits muscle growth. Myostatin was knocked out in mice by gene targeting and discovered to increase skeletal muscle mass two-fold and decrease body fat. Knock out mice also revealed significant hypertrophy and hyperplasia of skeletal muscle cells due to the myostatin

knockout (McPherron et al., 1997). Increasing the amount of muscle cells has the potential to cause dystocia problems in these species as the mammals will not be structurally sound to give birth to animals with such an increased size. Mutations of myostatin in domesticated cattle species including the Piedmontese, Belgian Blue, and Marchigiana, which produce the similar "double muscling" phenotype. Myostatin mutations and enhanced muscling also has been documented in Texel sheep and canine breed, Whippets, due to a two-nucleotide deletion in the gene (Rodgers et al., 2008). The deletion causes a premature stop codon, which results in the animal exhibiting the double muscling pheonotype. A premature stop codon in one copy of the gene also creates enhanced muscling, but not as significant as the two copy premature stop codon that creates the double muscling phenotype.

The mechanisms that regulate the activity of myostatin have yet to be exposed. Studies have been done to define the minimum domain necessary for inhibition of the myostatin homodimer (Jiang et al., 2004). Jiang et al. (2004) used a series of truncated GST-propeptide fusion proteins to find the inhibitory domain of the propeptide between amino acids 42-115. The N-linked glycosylation and intermolecular disulfide bonds were not necessary for inhibition, and the C-terminal (amino acids 99-266) was very stable, but did not express inhibitory activity. Research was also completed by Anderson et al. (2008) to reveal the predominate form of myostatin detectable in muscle as pro-myostatin, which constitutes the extracellular main pool of myostatin and can be cleaved by furin proteases. This result contrasts with previous findings that report cleaved prodomain and mature myostatin as the main pool of myostatin extracted from muscle lysates. The pro-myostatin interacts with latent transforming growth factor-β-binding protein (LTBP), especially LTBP-3 which is frequent in skeletal muscle and sequesters the pro-

myostatin in the extracellular matrix. The now conserved pro-myostatin limits myostatin signaling. This limitation in mouse muscle increases muscle fiber size.

It was found by Li et al. (2010) at the University of Hawaii that administered wild type and mutated propeptide decreased myostatin activity. The mutated propeptide showed an increase in growth performance by 11-15% (Li et al., 2010). In an attempt to block myostatin function, Kim et al. (2007) produced a polyclonal antibody against unprocessed chicken myostatin. The antibody, pAb0AVM46, showed a high affinity to the myostatin propeptide; however, the antibody showed very little affinity to the mature myostatin. The antibody was injected into the yolk of broiler eggs and by day three of incubation the embryos had reduced thigh and leg weight compared to the control or broilers from eggs with the same dose administered to the albumen. The high affinity of the antibody to the propeptide elevated the myostatin activity and decreased the muscle weight. This provides evidence that the propeptide inhibited the activity of myostatin in broilers (Kim et al., 2007).

Myostatin is key to understanding avian muscle development, as it is known to negatively regulate skeletal muscle growth. The primary objective of this study was to reveal myostatin isoforms of broiler and layer chickens and the heavyweight, random-bred, and lightweight lines of Japanese quail. Each line of inbred birds demonstrates differences in muscle mass. The heavy weight line was originally selected from the random-bred line at the Department of Animal Sciences Ohio Agricultural Research and Development Center (Reddish et al., 2003). The heavyweight line has more muscle mass than the random-bred or lightweight line. Therefore, it is hypothesized to have less myostatin or more myostatin inhibition by propeptide binding and inactivation, which will inhibit muscle growth and development. The myostatin isoforms are also compared by broiler and layer chickens during different stages of growth. Broiler chickens are

selected for improved growth rate and muscle mass compared to layer chickens. We hypothesize that because broilers produce so much more muscle mass, broilers will have more propeptide isoforms that bind and inhibit mature myostatin to promote increased muscle growth. Blood samples collected at various stages of embryonic development are tested to determine propeptide and mature myostatin during muscular growth. More cleavage and propeptide isoforms in the early developmental stages is indicative of increased muscle growth and development.

Materials and Methods

Animals

The Japanese quail used in this study are from weight-selected (HW), lightweight (LW), and random-bred (RBC) bred lines maintained at The Ohio State University Poultry Barn. Forty-eight broiler chicks were purchased with 48 layer chicks and hatched for the experiment. All birds were provided ad libitum access to food. Six broiler and leghorn chicks were used for muscle tissue and blood collection at each of the six time points as indicated in Table 1. Fertile eggs from each LW, RBC, and HW line were hatched and six birds were randomly collected at the six time points indicated in Table 2. At 0 d the LW line was 5.032 g, the RBC line was 5.842 g, and the HW line was 8.186 g. Collections were gathered until maturity at 49 d for broilers and leghorn chickens and 42 d for Japanese quail. Each specimen was randomly gathered. The quail at The Ohio State University show a significant difference between the three lines by maturity. The HW line develops more breast muscle as a percentage of body weight than the LW line.

Table 1. Broiler and leghorn collection time points.

Line	Day 2	Day 6	Day 10	Day 21	Day 35	Day 49
Broiler	n=6	n=6	n=6	n=6	n=6	n=6
Leghorn	n=6	n=6	n=6	n=6	n=6	n=6

Table 2. Japanese quail collection time points.

Line	Day 0	Day 4	Day 8	Day 15	D-25	Day 43
LW	n=6	n=6	n=6	n=6	n=6	n=6
RBC	n=6	n=6	n=6	n=6	n=6	n=6
HW	n=6	n=6	n=6	n=6	n=6	n=6

Tissue Collection

Each sample was euthanized by carbon dioxide (CO_2) method and exsanguination. Immediately following euthanasia, the bird was placed ventral side up, and an incision was made from below the breast muscle to each lateral side under the wing. Pectoral muscle from the breast was removed with forceps and scissors. Cardiac blood was obtained directly from the heart using a syringe and needle. The blood samples were collected before blood coagulation through a syringe, and the sample was slowly retracted and dispensed to prevent rupture of red blood cells and the release of protein. The samples were immediately snap frozen in dry ice for transportation and quickly stored at -80.0 ^{o}C.

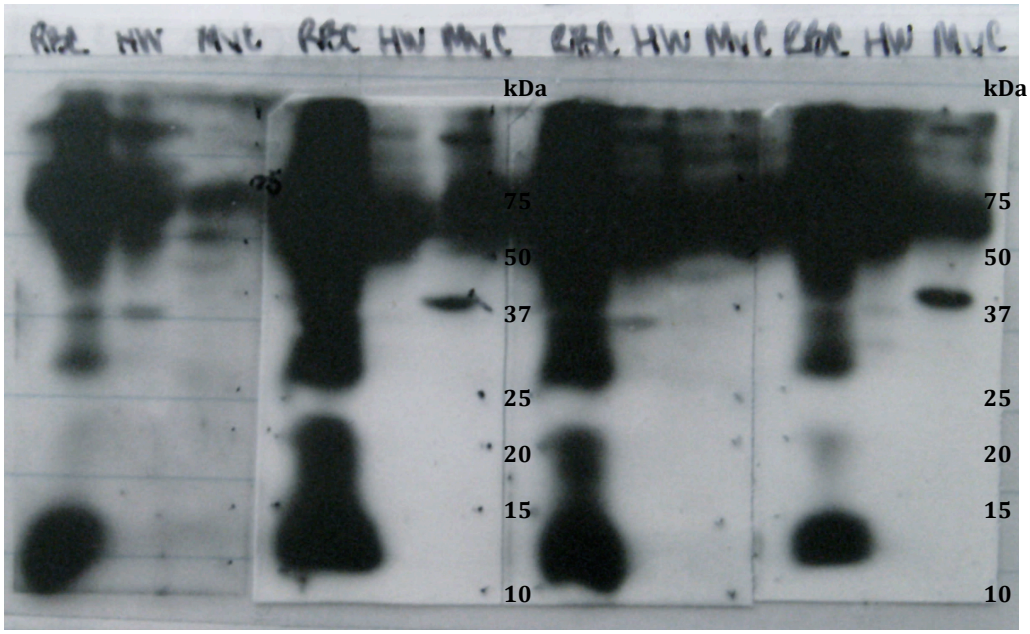
Tissue Analysis

The muscle tissue samples were homogenized before western blotting using a Tissuemiser homogenizer in lysis buffer (1% SDS) and combined with 2x Laemmli buffer. The

blood samples were diluted with a 1:8 dilution (1 part serum, 3 parts lysis buffer, and 4 parts loading buffer). The samples were separate by SDS-PAGE mini-Protean system. The protein was separated using 10% SDS-PAGE and transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 4% nonfat dry milk 0.5% bovine serum albumin for 30 min. The membrane was incubated in primary antibody (1:1,000 or 1:5,000 dilution, GDF8-1, RB 2697-1) for at least 1.5 h. The membrane was then washed 6 times in Tris-buffered saline-Tween at room temperature for 30 min and incubated in a secondary antibody (1:10,000 dilution, Jackson Immunity, 711-036-152) for 1 h. The membrane was washed 6 times in Tris-buffered saline-Tween at room temperature for 30 min. The protein was detected with ECL Plus (Amersham Biosciences) and exposure to x-ray film.

RESULTS:

The primary antibody was one of four antibodies designed in a previous experiment in this lab. The blood samples of the HW and RBC lines at maturity were tested between the four designed myostatin antibodies as indicated in Figure 1. The antibodies were derived from peptide synthesis and affinity purified. Antibodies 1 and 3 were from the same sequence, but different rabbits, and antibodies 2 and 4 were from a second peptide and different rabbits. Antibody MSTN-1 (GDF8-1, RB 2697-1) was chosen and used throughout the experiments because of the high binding affinity to the MYC positive control, without extra non-specific binding. The original secondary antibody showed high non-specific binding, so the Jackson Immunity antibody (711-036-152) was used throughout the experiments to remove extra non-specific binding. Bovine serum albumin was added to the 4% nonfat dry milk to remove common albumin binding proteins.



Antibody 4 Antibody 3 Antibody 2 Antibody 1

Figure 1. Western blot of HW and RBC line of Quail at 45 d to test four MSTN antibodies. Antibodies 2 and 4 were 1:500 concentration, and antibodies 1 and 3 were 1:5000 concentration. Myc tagged myostatin protein as a positive control. The dark binding in the RBC column appears when only the secondary antibody is used; therefore, the secondary antibody binds to quail antibody in the blood sample. The RBC sample has significant binding and may indicate higher myostatin binding in the RBC line.

β-mecaptoethanol (BME) was added to the loading buffer which breaks the disulfide linkage or propeptide binding according to Jin et al. (2004). Results were compared with RBC and HW lines without BME and with BME. Bands were displayed with BME around 37 and 15 kDa.

Western blotting was completed to compare Japanese Quail RBC and LW lines. Two samples from each collection day were used for each Quail line to compare the protein during the different stages of development. Figure 2 illustrates a comparison of Quail RBC and LW blood samples at each stage of development.

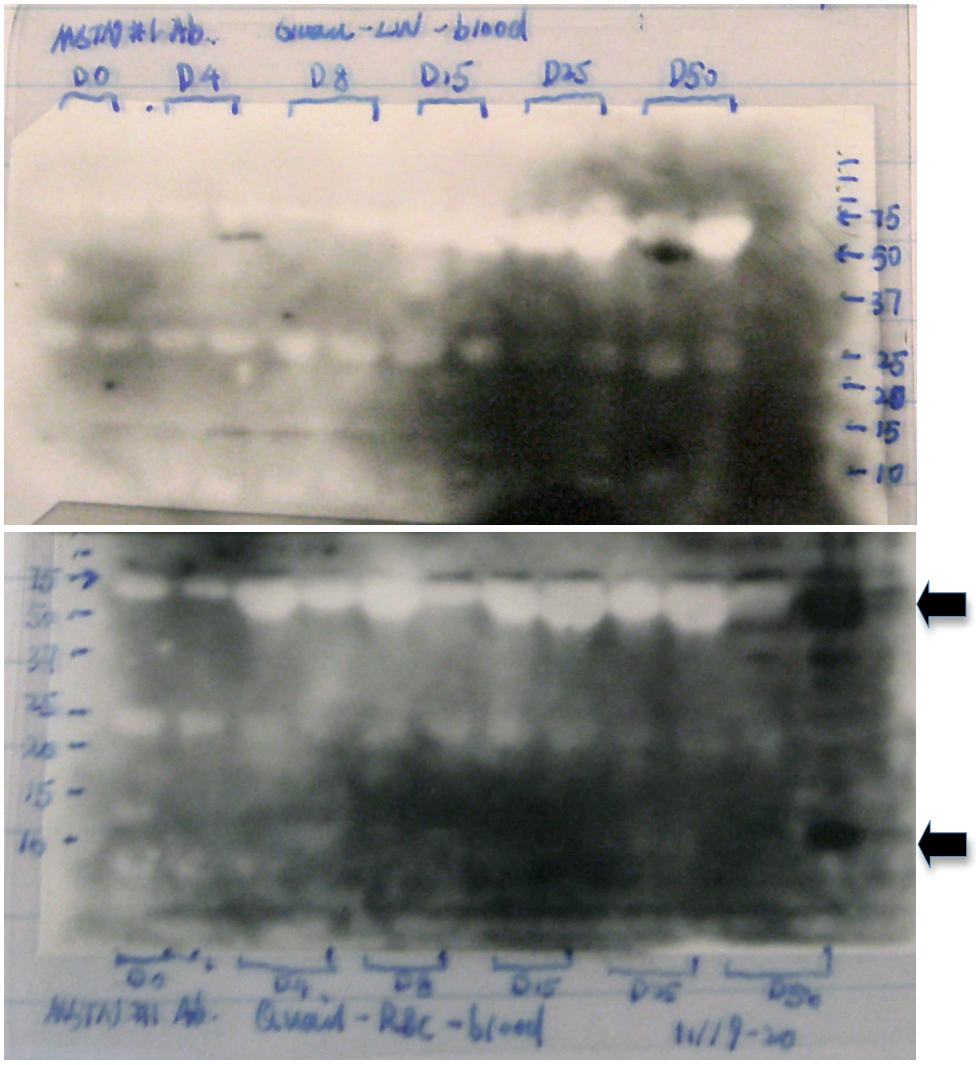


Figure 2. Western blot comparing Quail LW (a) and Quail RBC (b) blood samples through six stages of development, beginning with 0 d and ending with maturity at 50 d. MSTN-1 antibody was used with a 1:8 dilution of sample in a 10% gel. The arrows indicate significant binding at 37 and 10 kDa of 50 d indicating higher full length and cleaved myostatin in the mature sample.

The RBC film showed light binding specificity while the LW film lacked bands to indicate the antibody's specificity to the protein. To increase the protein, concentration was doubled from a 1:8 dilution to a 1:4 dilution. The antibody signaling was still low in the results, even with the

increased protein concentration. Coomassie staining of the membrane was completed to indicate the amount of protein loaded into the gel. Strong bands developed around 37 and 43 kDa for each sample, indicating propeptides. MYC and HA tag were used for a positive control.

DISCUSSION:

The aim of the study was to use western blotting to discover the myostatin isoforms during different stages of development of Japanese Quail, broilers, and layer chickens. The lack of MSTN-1 antibody binding specificity to the collected samples prevented the western blots from full development and definitive results. According to Moore (2009), Western blotting relies on the separation of proteins, transfer of proteins to a solid backing, and detection of a target protein by a matched antibody. The procedures succeeded with the samples during the separation and transfer of protein, so the problem was most likely detection of specific mysotatin isoforms by MSTN-1 antibody. The administered antibody works by sifting through the separated proteins to find the corresponding antigen to that specific antibody and binding to its receptors. A dark membrane blot will form on the membrane and show up on the film. According to Moore (2009), the antibody binds exactly to the correct antigen due to a small region of the antigen called the epitope, which binds to recognition sites on the arms of the antibody. The problem with this experiment lies in the binding of the epitope to the antibody. The results were not clear with consistent specific protein bands forming throughout the repeated procedures. The antibody could be binding to more than one epitope included in the sample, or the antibody may not have specific enough affinity to the myostatin epitope. Further research is required to fully understand which propeptide isoforms the antibody binds.

CONCLUSION:

The protein bands that formed indicate different isoforms are forming at different kDa in each period of development. A heavier formed band is indicative of glyosylation to the propeptide or glycosylation to a heavier isoform, while the small, light bands indicate the protein was cleaved multiple times or there was protein degradation. The multiple bands that formed at different kDa designate there was alternative splicing of the propeptide, and multiple isoforms are created throughout different stages of development. Research to understand which part of the propeptide the MSTN-1 antibody showed affinity to will indicate the separate isoforms formed through each stage the samples were collected. The results demonstrate our hypothesis that alternative splicing forms isoforms to bind active myostatin and increase growth at different stages of development; however, which isoforms are repeatedly formed is still unclear. Additional research that results in consistent binding of antibody to the proteins is necessary to determine the role of myostatin isoforms during development.

IMPLICATIONS:

The future population boom and subsequent food quandary is pushing industries to find new ways to produce high quantities of food with less space. There is an increased concern to improving animal production in the most efficient manner to support the world. Scientists, engineers, and producers combine to find the most effective solutions and the opportunity to understand muscle development within avian species, which will benefit the world population.

The author would like to recognize Yan Song and Dr. Kichoon Lee for their contributions to the research.

REFERENCES

Anderson, S. B., Whitman, M., & Goldberg, A. L. (March 14, 2008). Identification of a novel pool of extracellular pro-myostatin in skeletal muscle. Journal of Biological Chemistry, 283, 11, 7027-7035.

Jiang, M. S., Liang, L. F., Wang, S., Ratovitski, T., Holmstrom, J., Barker, C., & Stotish, R. (January 01, 2004). Characterization and identification of the inhibitory domain of GDF-8 propeptide. Biochemical and Biophysical Research Communications, 315, 3, 525-31.

Jin, H.-J., Dunn, M. A., Borthakur, D., & Kim, Y. S. (January 01, 2004). Refolding and purification of unprocessed porcine myostatin expressed in Escherichia coli. Protein Expression and Purification, 35, 1, 1-10.

Khan, S., & Hanjra, M. A. (April 01, 2009). Footprints of water and energy inputs in food production - Global perspectives. Food Policy, 34, 2, 130-140.

Kim, Y. S., Bobbili, N. K., Lee, Y. K., Jin, H. J., & Dunn, M. A. (June 01, 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. Poultry Science, 86, 6.

Li, Z., Zhao, B., Kim, Y. S., Hu, C. Y., & Yang, J. (January 01, 2010). Administration of a mutated myostatin propeptide to neonatal mice significantly enhances skeletal muscle growth. Molecular Reproduction and Development, 93, 1, 76.

McPherron, A. C., Lawler, A. M., & Lee, S. J. (January 01, 1997). Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. Nature, 387, 6628, 83-90.

Moore, C. (2009). Introduction into western blotting. Retrieved from http://static.abdserotec.com/Lit-pdfs/Brochures1/westernblotbook.pdf

Reddish, J. M., Nestor, K. E., & Lilburn, M. S. (January 01, 2003). Effect of selection for growth on onset of sexual maturity in randombred and growth-selected lines of Japanese quail. Poultry Science, 82, 2, 187-91.

Rodgers, B. D., & Garikipati, D. K. (August 01, 2008). Clinical, agricultural, and evolutionary biology of myostatin: A comparative review. Endocrine Reviews, 29, 5, 513-534.