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Sexually dimorphic development of chicken breast muscle

Yihan Hu

Student Exam Number: B100977

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Supervisors: Dr. Michael Clinton and Dr. Tom Burdon

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DECLARATION

(a) that the thesis has been composed by the student, and

(b) the work is the student's own, and

(c) that the work has not been submitted for any other degree or professional qualification except as specified

Signature:

ABSTRACT

Adult male chickens have a greater muscle mass than adult female chickens. Available data suggests that this dimorphism is a result of events that occur during embryonic myogenesis. Previous results from our laboratory have established that at day 12.5 of embryonic development (E12.5), male embryos have a greater mass of breast muscle than female embryos, but that there is no significant difference in male and female muscle mass at other stages of development. The objective of this research was to characterise morphological difference between male and female embryonic muscle and identify possible factors involved in this process. Here, we have further characterised this E12.5 morphological difference, and found that male myofibres are larger than female myofibres but myofibre density and PAX7 positive cell density is lower in males than in females. We also measured the expression level of MuSK, a key gene in neuromuscular junction formation, and found that MuSK is expressed at higher levels in male chicken breast muscle than in female chicken breast muscle. This may result in a greater degree of neuromuscular junction formation and lead to more myofibres surviving in male embryos.

Keywords: Sexually dimorphism, myogenesis, myofibre, neuromuscular junction, MuSK

LAY SUMMARY

Adult male chickens have greater muscle mass than adult female chickens: a fact that has great significance to poultry meat production. It is thought that muscle fibre number is determined at hatch and that muscle growth post hatch is the result of hypertrophy (increase in cell size). Consequently, the sexual dimorphism of adult muscle mass is thought to be a result of events occurring during embryonic development, although the details of the underlying mechanisms remain unknown. Previous results from our lab established that at day 12.5 of embryonic development (E12.5), male embryos have a greater mass of breast muscle breast muscle than female embryos. Here we have further characterised this morphological difference, and measured the muscle fibre number, muscle fibre cross-sectional area and fetal myoblasts numbers in male and female chicken embryos at E12.5. Survival of myotubes/myofibres is known to be dependent on motor neurone innervation and preliminary data suggests that expression level of MuSK, a key gene in neuromuscular junction formation, is higher in male skeletal muscle than female skeletal muscle during embryonic development. We have measured the expression levels of MuSK and other genes involved in motor neuron or muscle development from E6.5 to E16.5 to try and identify factors involved in the sexual dimorphic development of chicken breast muscle. As MuSK is also reported to stimulate BMP signalling in muscle, and given that BMP signalling is a key element in muscle development, we also investigated the downstream transcription factors of BMP.

1.0 INTRODUCTION

1.1 Poultry meat production

The chicken is now a major meat source globally. The history of raising chickens for foodstuffs dates back thousands of years. The domestic chicken plausibly originates from the hybridization of red jungle fowl and grey jungle fowl in south Asia (Eriksson et al., 2008). Gradually, chickens became increasing popular in human society and spread worldwide due to the simple breeding requirements. In modern society, the chicken still plays an important role in the human diet. The increasing demands of high quality protein makes chickens breast the most economically valuable part in the chicken, which contains more protein and moisture but less fat (Cozzolino et al., 1996). The poultry industry has made strenuous efforts to increase the quantity as well as quality of chicken meat, by screening chicken lines with greater growth rates and higher feed conversion ratio (FCR). This kind of selection makes muscle fibre number and cross section area in fast growth lines significantly higher than slow growth lines (Dransfield et al., 1999). However the mechanisms involved in this process are still unclear.

1.2 Sexual dimorphism in chickens

Male and female chickens have a large difference in appearance: in addition to reproductive and decorative difference, the male chicken is much heavier with a greater muscle mass than female chicken. Elucidating the mechanism that result in the sexual dimorphism seen in chicken breast muscle could be of enormous economic benefit to poultry industry, and contribute significantly to the

sustainability of food production.

Avian sex is clearly chromosomally based: the male chicken is homogametic (ZZ) as regards sex chromosomes while the female chicken is heterogametic (ZW). Although the sex determination mechanism in chicken has not been elucidated, it clearly different from that found in mammals. In the classical mammalian model, a single testis-determining gene called Sex-determining region Y (SRY) (Sekido and Lovell-Badge, 2009) triggers male sex determination. SRY was identified as a conserved region located on the male-specific Y chromosome in human and mice (Berta P et al., 1990). Researchers found that SRY is sufficient to induce male development when introduced into chromosomally female (XX) mouse embryos (Koopman and Gubbay, 1991). SRY binds a gonad-specific enhancer of the autosomal gene SOX9 in mice and upregulates SOX9 expression level (Sekido and Lovell-Badge, 2008) which is crucial for male development. Even in the absence of SRY, SOX9 expression in the undifferentiated XX gonad can leads to sex reversal (Vidal et al., 2001). Although a similar male-specific SOX9 expression pattern is observed in chicken gonads (Kent et al., 1996), to date, no Sry-like single sex-determining gene has been found.

Two credible sex determining mechanisms have been proposed for birds. One is the existence of an ovary-determining gene on the W chromosome in the female chicken and another is a dosage effect dependent on the two Z chromosomes in the male and the single Z-chromosome in the female (Clinton, 1998). Up to now, DMRT1 (doublesex and mab-3-related transcription factor 1) is the strongest candidate for a male sex-determining gene in birds, under the dosage theory (Nanda et al., 2000). Knock down of DMRT1 expression in ovo results in the feminization

of the gonads in genetically male (ZZ) chicken embryos, activation of the ovarian marker aromatase, and a decline in the testicular marker SOX9 in the left gonad (Smith et al., 2009). Currently there are no plausible candidates for a W-linked ovary-determining gene (Smith et al., 2003). However, further investigation is required to determine whether there is another gene involved upstream in the male-determination pathway as well as considering other alterative potential W-linked ovary-determining genes.

1.3 Cellular autonomy

In addition to the sex determination mechanism differing in birds and mammals, the factors that determine the sexual phenotype also differ between these species. The sexual phenotype is usually regarded as being determined by hormones, based on studies conducted in mammals. However, in recent years, it has become widely accepted that cell autonomous sex identity (CASI) also plays a role in phenotype determination in birds (Arnold and Chen, 2009; Zhao et al, 2010; Ngun et al., 2011). While hormones are still considered as the dominant determining factors for somatic phenotype in mammals, in birds, a different theory has been proposed. This theory arose through the observation of rare gynandromorph birds (Figure 1-1), that occur naturally and where one side looks male and another side looks female (Zhao et al., 2010). Our laboratory was able to demonstrate that the side that appears male is composed predominantly of genetically male cells (ZZ), while the side appears female is composed predominantly of genetically female cells (ZW). Given that both sides are exposed to the same profile of hormones, the most likely explanation for this bilateral sexual dimorphism, is that nature of the cell determines the phenotype (Zhao et al., 2010). Research using transplantation studies to generate

embryo with chimeric gonads further confirmed that the sexual phenotype was dependent on cell autonomous sexual differentiation in birds. GFP marked cells from female donors were not incorporated into host male-specific testes structures, and GFP marked cells from male donors were not incorporated into female-specific ovarian structures (Zhao et al., 2010). This suggests that sex identity in avian gonads cells is cell autonomous rather than decided by the hormones. Interestingly, not only the gonads, other tissues like muscle also shows such cell autonomous sexual differentiation (Henry and Burke, 1999; Bruggeman et al, 2002). Sex reversal by *in ovo* injection of testosterone did not increase the muscle weight in female chicken (Henry and Burke, 1999), which contrast with the classical mammal model. The failure of sex reversal treatment indicates that a resistance exists in the chicken breast muscle against the gonadal hormone. In conclusion, CASI plays a more important role than hormones in determining tissue phenotype, in birds.

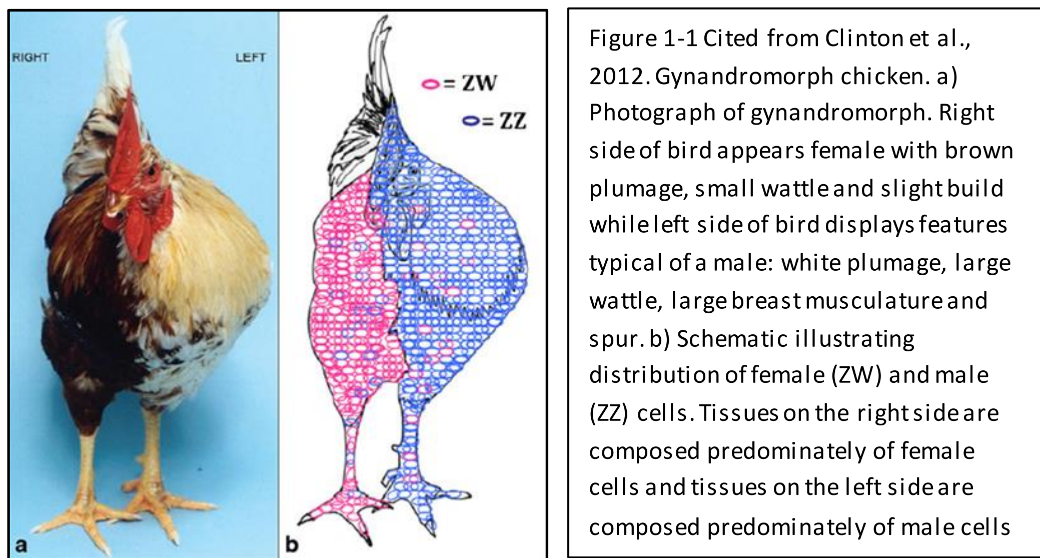

Figure 1-1 Cited from Clinton et al., 2012. Gynandromorph chicken. a) Photograph of gynandromorph. Right side of bird appears female with brown plumage, small wattle and slight build while left side of bird displays features typical of a male: white plumage, large wattle, large breast musculature and spur. b) Schematic illustrating distribution of female (ZW) and male (ZZ) cells. Tissues on the right side are composed predominately of female cells and tissues on the left side are composed predominately of male cells

1.4 Skeletal muscle development

1.4.1 Hyperplasia

Muscle is derived from the mesoderm in a process called myogenesis (Stockdale, 1992). Muscle growth can be divided into two 'types': hyperplasia and hypertrophy. Hyperplasia relates to the increase in muscle cell number and is confined to embryonic stage myogenesis. During the period of hyperplasia, myogenesis involves two critical phase: primary myogenesis and secondary myogenesis (Swartz et al., 1994).

Primary myotubes form during primary myogenesis. Embryonic myoblasts, a type of embryonic progenitor cell derived from the somites, differentiate into myocytes (Campbell, 1999), and then, a number of myocytes attach to each other and fuse to form primary myotubes. Primary myotubes have centrally located nuclei (Abmayr and Pavlath, 2012).

Secondary myotubes form during secondary myogenesis and constitute most of the myofibres in adult muscle (Duxson and Usson, 1989). During this period, fetal myoblasts fuse together to form the secondary myotubes (Swartz et al., 1994; Messina and Cossu, 2009; Fredette and Landmesser, 1991). All the secondary myotubes overlap the endplate region of primary myotubes (Duxson et al., 1989), with the result that the position of secondary myotubes is highly dependent on the position of primary myotubes. In fact, secondary myotubes form on the scaffold of primary myotubes.

It remains likely but uncertain that the formation/survival of secondary myotubes depends on innervation. Freddete and Landmesser suggest that secondary myotube

cannot form without innervation (Fredette and Landmesse, 1991), although this is challenged by Ashby (Ashby et al., 1993). In this study by Ashby an aneural mouse which lacks the peroneal nerve had a normal number of secondary myotubes in the hindlimb compared with a wild type mouse, although the muscle underwent atrophy later in development (Ashby et al., 1993).

During secondary myogenesis, primary myotubes are coupled with secondary myotubes under the same basal lamina. On maturation, secondary myotubes no longer associate with primary myotubes and develop their own basal lamina (Swartz et al., 1994). At the same time, bundles of myofibres accumulate together and forms muscle fascicles, surrounded by the perimysium. Figure 1-2 shows the general structure of adult muscle.

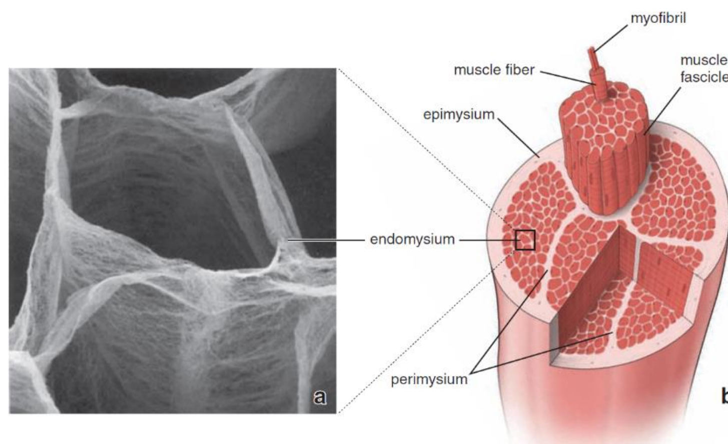

Figure 1-2 Cited from Ross and Pawlina, 2015.General organization of skeletal muscle. a. This freeze fracture scanning electron micrograph of an intramuscular connective tissue was obtained from the bovine semitendinous muscle. The specimen was routinely fixed for SEM and subsequently treated according to the cell maceration method with sodium hydroxide to remove muscle cells. Note a delicate honeycomb structure of the endomysium surrounding individual muscle cells. x 480. b. This schematic diagram shows the general organization of skeletal muscle and its relation to the surrounding connective tissue. Note the organization of the endomysium that surrounds individual muscle cells (fibers), the perimysium that surrounds a muscle bundle, and the epimysium that surrounds the entire muscle

Above all, hyperplasia results in an increase in myofibre number, but this is restricted to embryonic development and ceases at hatch, with the result that muscle

fibre number is set and no longer increases post-hatch (Smith, 1963).

1.4.2 Hypertrophy and satellite cells

Muscle growth post-hatch is due to a process known as hypertrophy, which result in an increase in the size of individual muscle fibres. This process involves the fusion of satellite cells to existing fibres. These are multipotent muscle stem cells that arise from the de-differentiation of myoblasts and which do not form muscle fibres (Zammit et al, 2006). In order to raise the protein synthesis level during post-hatch myogenesis, muscle fibres needs the increased transcription and translation activity of additional nuclei, and this is achieved by the fusing of satellite cells to existing fibres. In this process, satellite cells undergo differentiation and fusion that is similar to that seen with the myoblast druing embryonic muscle development (Zammit et al, 2006). Isotope-labeled H-thymidine in satellite cells given to rats proves the fusing of satellite cells in muscle, as labeled satellite cells were then found in the muscle fibres in a few hours (Moss and Leblond, 1971). It has been reported that satellite cells make up the majority of nuclei in mature muscle fibres (Allen and Merke, 1979). The differentiation and fusion of satellite cells greatly increase the myofibre size, and is the basic of hypertrophy during posthatch myogenesis. Satellite cells are not limited to the process hypertrophy, but also function in the repair and regeneration of muscle, and are crucial to the maintenance of muscle during adulthood (Charge and Rudnicki, 2004).

Currently, PAX7 (paired box 7) is the most reliable marker of satellite cells in adult muscle (Seale et al., 2000; Oustanina et al., 2004; Zammit et al, 2006), where it is

used to identify quiescent satellite cells: the most common type found in mature muscle (Schultz et al., 1978). After activation, satellite cells start to express MyoD (Myogenic Differentiation) (Füchtbauer and Westphal, 1992), co-expressing PAX7 until they start differentiation and fuse into myotubes (Zammit et al, 2006).

There is no unique molecular signature to distinguish satellite cells from other myoblasts during embryonic development (Zammit, 2008), and satellite cells cannot be classified by anatomical characters until the end of fetal development (Kelly and Zacks, 1969; Ontell and Kozeka, 1984; Zammit, 2008). During embryonic genesis, PAX is expressed in several muscle progenitor cells (Figure 1-3). A population of skeletal muscle progenitor co-express PAX3 and PAX7 but no myogenic marker was identified during embryonic myogenesis. (Kassar-Duchossoy et al., 2005; Relaix et al., 2005). This population of Pax positive cells are not only the progenitor of fetal myoblasts, but also the progenitor of satellite cells (Schienda et al., 2006).

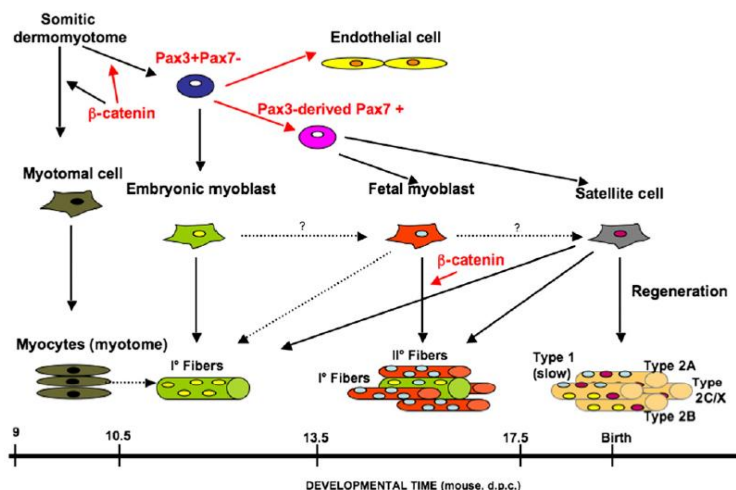

Figure 1-3 Cited from Messina and Cossu, 2009. A scheme of the possible lineage relationships of skeletal myoblasts (and the role of b-catenin). The steps identified by the present study are shown in red.

1.4.3 Embryonic muscle weight on Embryonic Day 12.5 (E12.5)

During embryo development, both body weight and muscle weight increase. During embryogenesis, myofibre number is the major determinant of muscle size. Myofibre number in chicken breast is reported to show a sharp increase between E10 to E16, but then shows no significant increase in the subsequent stages up to hatch E21(Kikuchi et al., 1973). Previous work carried out by Hanafi Sulong in our laboratory shows that embryo body weight also increases significantly between E8.5 to 16.5, but that there is no statistically significant difference between body weight of male and female chicken embryos during this period (Figure 1-4) (Sulong, 2016). These findings are supported by other researchers (Coleman et al, 1964; Marks, 1985). Sulong also measured the breast muscle weight of male and female embryonic chicks from E8.5 to E16.5, and found that at E12.5 male chicken breast muscle is significant heavier than female chicken breast muscle. This difference was not evident at the other stages studied (Figure 1-5) (Sulong, 2016). These measurements were repeated on a further three sets of male and female breast muscles collected at E12.5, with similar results. In each case, the weight of muscle was estimated for 10 male and 10 female embryos.

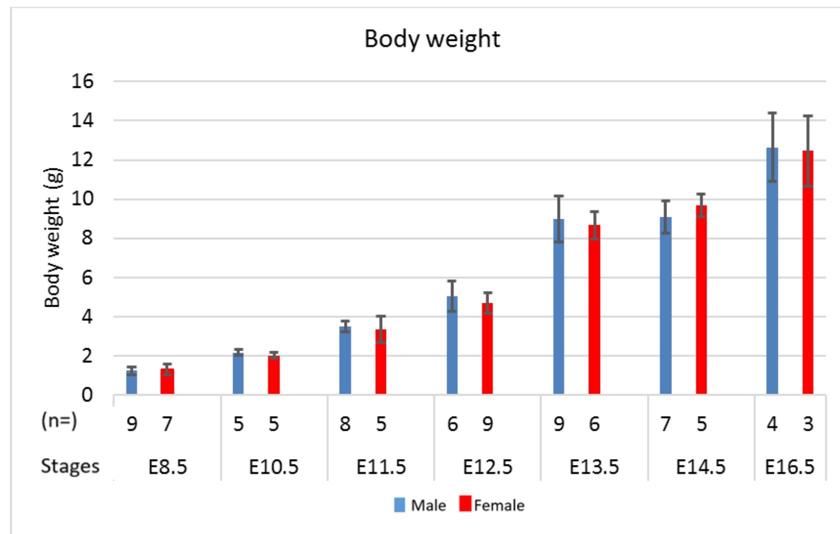

Figure 1-4 Cited from H Sulong, 2016. Relative comparison of mean body weight at different stages of embryonic development. Histogram represent mean weight ± 1.S.D. "n" refers to biological replicates.

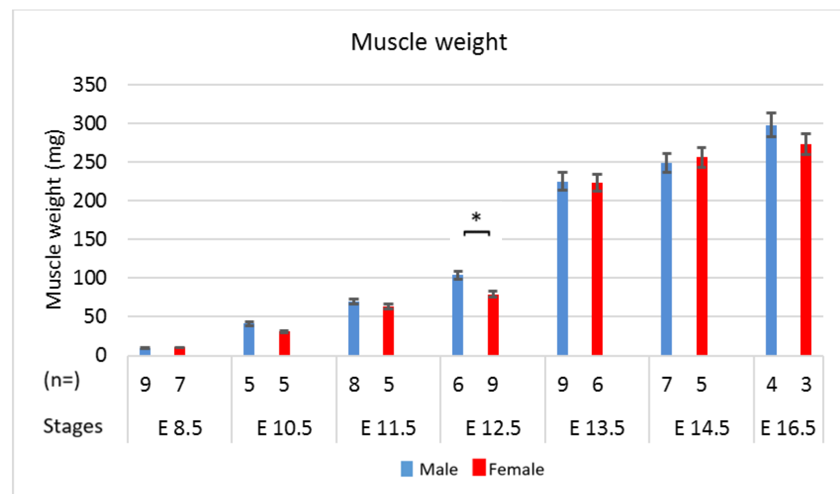

Figure 1-5 Cited from H Sulong, 2016. The relative comparison of muscle weight at different stages of embryonic development . Histogram bars represent mean weight ± 1.S.D. "n" refers to biological replicates.

1.5 MuSK and neuromuscular junction formation

The neuromuscular junction (NMJ) is a chemical synapse between a muscle fibre and a motor neuron (Levitan et al., 2015), and plays a significant role in the survival of both muscle fibre and motor neuron (DeChiara et al., 1996). Through the NMJ, muscle fibres are innervated by the motor neurons (Hall and Sanes, 1993). Important chemical signals are transmitted, released from the presynaptic vesicles. One such neurotransmitter is acetylcholine, and this combines with acetylcholine receptors (AchRs) which are ligand-gated ion channels on the muscle fibre and causes depolarization of the muscle fibre and contraction. Other neurotrophic and myotrophic factors may also be exchanged through the NMJ as well. (DeChiara et al., 1995; Helgren et al., 1994)

The formation of the NMJ is a result of numerous interactions between the motor neuron and the muscle fibre. However, muscle-specific kinase (MuSK), a member of the receptor tyrosine kinases (RTKs), is the key factor in NMJ formation (DeChiara et al., 1996; Okada K et al., 2006). MuSK forms a receptor complex with agrin which is a nerve-derived factor that induces the clustering of AchRs via rapsyn and Dok-7 (Okada K et al., 2006; Glass et al., 1996). A previous RNA-seq analysis carried out by our laboratory showed that there is a difference in MuSK expression between male and female chicken embryonic breast muscle, suggesting that MuSK may play a role in influencing sexually dimorphic development in chicken.

1.6 BMP signalling and MuSK in muscle development

In addition to the established role in NMJ formation, the muscle-specific MuSK has also been implicated in BMP signalling. Bone morphogenetic protein (BMP) signaling is a basic signaling pathway utilized in almost all the tissues during development, and has been shown to play a role in regulating muscle mass (Sartori et al., 2013). BMPs are cytokines belonging to the transforming growth factor-β (TGFβ) superfamily. There are two different classes of BMP receptors. BMP type I receptors which include ALK1, ALK2, ALK3, ALK4 and ALK6, and type II receptors which include BMPRII, ActRIIa, and ActRIIb (Nohe et al, 2004). BMPs bind the receptors on the cell surface, and this leads to the downstream phosphorylation of SMAD 1/5/8 (small mothers against decapentaplegic). This complex then associates with SMAD4, and enters the nucleus and act as a transcription factor. This regulates a wide variety of genes inducing those involved in skeletal muscle growth (Figure 1-5) (Bragdon et al., 2011; Winbanks et al., 2013). BMP activity can also protect the muscle from atrophy caused by denervation (Winbanks et al., 2013). It was later found that BMP signaling reduces denervation-induced wasting by inhibiting an uncharacterized F-box protein (Fbx0-30) called MuSA1 (muscle ubiquitin ligase of SCF complex in atrophy-1) which labels protein for degradation (Sartori et al., 2013).

MuSK was recently reported as a co-receptor of BMP signaling (Yilmaz et al., 2016). MuSK binds to the ALK3, ALK4, ALK6, rather than type II receptors, and enhances the BMP-induced expression (Yilmaz et al., 2016). It is not known whether the role MuSK plays in the neuromuscular junction intersects with any role MuSK may plays in BMP signaling pathway.

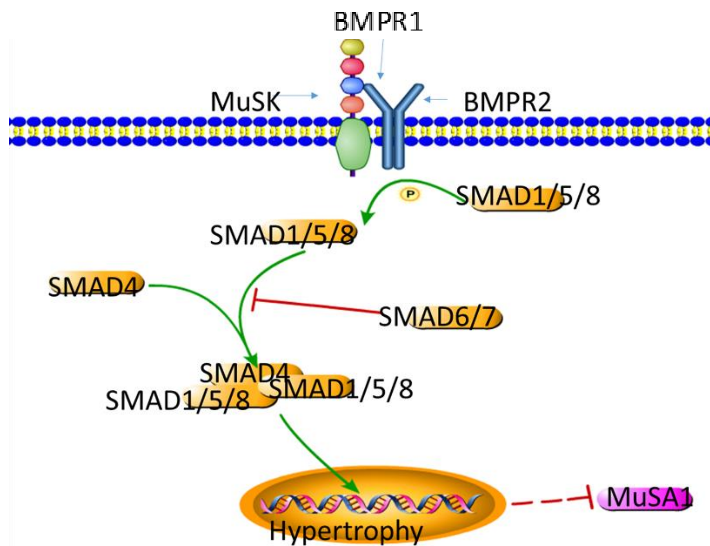

Figure 1-5 MuSK is the co-receptor of BMP signalling pathway. Transcription factor SMAD1/5/8 is phosphorylated downstream of BMP signalling. Phosphorylated SMAD1/5/8 recruits SMAD4 to form a complex and then enters the nuclei.

1.7 Project aim and objectives

It has been shown that the differences between adult male and female chicken muscle are mainly due to differences established during embryonic development. However, there is no difference in male and female body weight during development, and differences in breast muscle weight are only obvious at E12.5. The CASI theory leads us to expect differences in sex chromosome gene expression in male and female breast muscle, and MuSK is a strong candidate to play a role in the development of this sexually dimorphic tissue. The main objectives of this project are: 1) to characterize muscle morphology in male and female embryos at E12.5, 2) assess whether MuSK is likely to play a role in the sexually dimorphic development of chicken breast muscle, and 3) determine whether any role played by MuSK relates to BMP signalling or not. To address these issues, this project is

divided into three parts 1) Measure the cross-sectional area and myofibre number in male and female breast muscle on E12.5, and determine the number of PAX7 positive cells in male and female muscle. 2) Measure MuSK expression levels in male and female breast muscle from E8.5 to E16.5. 3) Investigate the role of BMP signaling in the sexually dimorphic development of chicken breast muscle.

2.0 MATERIALS AND METHODS

2.1 Eggs incubation

Fertile chicken eggs (Gallus gallus), were collected from the National Avian Research Facility at the Roslin Institute, Easter Bush, UK, and incubated at 37-38.5 °C and 37 % humidity for defined periods. Following incubation, eggs were stored in a cold chiller at 15°C to slow development, and help to synchronise dissected tissues.

2.2 Chicken breast muscle dissection

Embryos were removed from shells, blotted dry and placed in pre-weighed plastic Petri dishes. Prior to dissection, embryos were killed by decapitation, and body weights were measured and recorded. Prior to, and between dissections, forceps and scissors were washed in water, 70% ethanol and PBS to minimise cross contamination. Dissection scissors were used to cut a slot in the skin layer and expose the breast muscle. In this way, the border of breast muscle can be clearly identified. The breast muscle was separated from the embryo by cutting along the border and underneath the muscle. For embryos younger than E14.5 left and right breast muscles were combined for storage/processing. For embryos later than E14.5, left and right breast muscles were collected and stored separately. The viscera was then removed and the embryos sexed by visual examination of the gonads. Sexing was confirmed by Invader assay using DNA extracted from a small piece of the limb tissue (around 50mg).

2.3 W-Repeat Invader Sexing Assay

The Invader Sexing Assay (Clinton et al., 2016) was used to confirm/determine the sex of individual chicken embryos. Approximately 50mg of tissue was collected into

one hundred microliters of autoclaved water. The sample tubes were then incubated at 95°C for 5 minutes to lyse the cells and to release the genomic DNA, and then placed on ice for 5 minutes. Tubes were then vortexed at the maximum speed for 45 seconds. A 2x Invader master mix was prepared: each single reaction contains 3.5µl of FRET mix, 3.0µl of Probe, 1.0 µl of Cleavase enzyme. Seven and half microliters of the master mix was dispensed into individual wells of a 96-well qPCR plate (Abgene 0990), followed by the same volume of the solution containing sample DNA. The wells were sealed and the plate was centrifuged at 2000 rpm for 2 minutes. Following centrifugation, the plate was placed in a Stratagene Mx3000P qPCR machine. The program was set to incubate the plate at 63°C, and record the FAM and ROX fluorescence signal every minute for a total of 60 minutes. Wells containing DNA from known male and female birds were included as positive-Controls, and wells containing water were included as negative-Controls.

2.4 Immunostaining and microscopy

2.4.1 Tissue embedding and optimization

For each embryonic stage examined, the dissected breast muscle (5 male and 5 female) was placed in 4% paraformaldehyde (PFA) - PBS solution (pH 7.2, Sigma-Aldrich®) and incubated at 4°C overnight. After fixation in PFA-PBS solution, the tissues were washed once with PBS, and then incubated in 0.12M Phosphate buffer-15% sucrose-PBS (Sigma-Aldrich®) for either a few hours or overnight. The muscle tissues were then transferred into pre-heated 0.12M Phosphate buffer-15% sucrose-7.5% gelatine at 42°C (Sigma-Aldrich®) and incubated at 42°C for 30-60 minutes. Sucrose-gelatine solution (1.5ml) was dispensed into a 2cm x 2cm plastic weighing boat, distributed

evenly by gentle shaking and cooled to form an even gel layer. Muscle samples were placed on the gel surface, and then submerged in sucrose-gelatine solution before setting at 4°C.

Under a dissecting microscope, pairs of breast muscle from individual embryos were divided into four portions: Anterior left and right (LA, RA) and posterior left and right (LP, RP). LA and RA portions were then placed side-by-side on a cork disc and mounted in O.C.T.™(Tissue-Tek®). Samples were then placed in isopentane pre-cooled in the dry ice, and embedded tissues stored at -80°C until cryosectioning.

2.4.2 Cryosectioning

For cryosectioning, the chamber temperature of an OTF5000 cryostat (Bright Instruments) was set at -28°C and the temperature of the specimen holder was set at -23°C. Embedded muscle tissue was removed from the cork disc and affixed to a metal chuck using O.C.T. The knife and specimen were equilibrated to the chamber temperature for approximate 20 minutes. Next, 10 µm sections were cut, and sections were placed on glass slides (SuperFrost Plus, Thermo Fisher Scientific). Sequential sections were placed on 10 slides in order, repeated for further sections and each slide contained between 4 and 8 sections: for example, slide number 1 contained sections number 1, 11, 21, 31, 41, 51, 61 and 71. In this way, the sections on each slide represented a large area of the sample, from the beginning to the end. The slides were allowed to air dry for 30 minutes. After all the sections were collected, the slides were stored in slide boxes at -80°C.

2.4.3 Immunohistochemistry of chicken myofibres

Slides containing sections were washed in phosphate buffer solution (PBS) at 37°C for 20 minutes with at least one change of PBS. A final wash was carried out by using PBSTr (0.3% Triton™ X-100 (Sigma-Aldrich®)-PBS), rocking gently. Blocking buffer was prepared with 10% donkey serum (Sigma-Aldrich®), 1% bovine serum albumen (Sigma-Aldrich®) and PBSTr. Wash solution was then removed and slides placed horizontally in a humid chamber. Blocking buffer (240 µl) was added to each slide and slides left at room temperature for 1 hour. The blocking buffer was then removed and replaced by 240µl of primary antibody (Laminin anti-rabbit 1:500 (Sigma-Aldrich® L9393), PAX7 anti-mouse 1:500(R&D® MAB1675)) diluted in blocking buffer. The slide chamber was incubated overnight at 4°C. To help restrict the antibody solution to the area of the sections, the slide can be tilted slightly, or a PAP pen can be used to draw a circle around the sections. After overnight incubation, the primary antibody solution was carefully tapped off, and the sections washed in PBSTr at room temperature for 30-60 minutes with at least 3 changes. After draining slides, 240 µl secondary antibody (Dk-Rb-546 1:500, Dk-Ms-488 1:500 (Alexa Fluor®)) diluted in blocking solution was added and the slides incubated for 1-2 hours at room temperature in the dark (light exposure was limited for the remainder of the procedures). The slides were then washed in PBSTr for 30-60 minutes with at least 3 changes, rocking gently. Hoechst33342 (Sigma-Aldrich®) diluted in blocking solution was added and slides incubated for approximate 5 minutes. The slides were then washed in PBSTr for 10 minutes with at least 3 changes. Invitrogen Prolong Gold mountant was used for permanent mounting, then the slides were covered with

coverslips. The slides were stored temporarily at 4°C temporarily, and long-term at -20°C.

2.4.4 Microscopy

A haemocytometer was used to accurately scale images. In order to estimate myofibre number, myofibre cross-section area (CSA) and number of PAX7 positive cells, 5 images of each section (from five male and five female muscles) were captured under 400X magnification using a Leica DMLB upright Fluorescent microscope. For analysis, individual images were randomly assigned a number between 1 and 50 by an independent staff member and estimates of CSA, fibre number and cell number completed 'blind'. Following analyses, image identity was decoded to generate mean values.

2.5 QPCR analysis

2.5.1 RNA extraction

Dissected muscle tissues (5 male and 5 female for each stage) were placed in labelled tubes and flash-frozen in the liquid nitrogen. After all the muscle tissues were collected, tubes with muscle were stored at -80°C until RNA extraction. A Polytron Homogenizer was used to homogenize the muscle tissues in Bijou tubes containing 3 ml of RNA-Bee™ (Tel-Test Inc. ®) solution (3 x 15 seconds at full speed). Between samples, the Polytron probe was washed extensively in, sequentially, sterile water, 0.2M NaOH and sterile water, to prevent cross-contamination of samples. The homogenate was divided into 2 tubes and 0.3ml of Chloroform was added to each 1.5 ml of homogenate. Tubes were shaken vigorously for 15 second, incubated on ice for

5 minutes and then centrifuged at 12000 g for 15 minutes. The aqueous phase was transferred to a new tube and an equal volume of 2-isopropanol was added. Following a gentle mixing, samples were incubated at room temperature for 10 minutes, and then centrifuged at 12000 g for 15 minutes. The resulting RNA pellet was then washed with 1 ml of 75% Ethanol (at -20°C), the supernatant carefully discarded and the pellet dried at 37°C for 5 minutes. The RNA pellet was dissolved in 45 µl RNase free water on ice for 10-20 minutes and RNA aliquots combined. 10 microliters of 10X TURBO DNase Buffer, and 2 µl TURBO DNase was added to each sample and incubated at 37°C for 20-30 minutes. Five microliters DNase I inactivation Reagent was added, mixed well and incubated at room temperature for 2minutes. After centrifugation at 12000 g for 15 minutes, the RNA solution was carefully transferred to a new tube, precipitated by adding 10 µl of 3 M NaOAC, and 250µl of Ethanol and stored at -80°C overnight. On the next day, RNA pellet was centrifuged down and washed with 100 µl 75% Ethanol (at -20°C). The pellet was dried at 37°C for 5 minutes and dissolved in 20-200 µl RNase free water on ice for 10-20 minutes. Quality and quantity of RNA was assessed by NANODROP and Agilent Analyzer.

2.5.2 Agilent analysis

Five hundred and fifty microliters of 'stock' RNA 6000 Nano gel matrix (Agilent Tech®) was pipetted into a spin filter and centrifuged at 1500g for 10 minutes at room temperature. The filtered gel was divided into 65 µl aliquots for routine use. For individual runs, RNA 6000 Nano dye concentrate was equilibrated to room temperature for 30 minutes, then vortexed for 10 seconds and briefly centrifuged. One microliter of dye was added into 65 µl aliquot of filtered gel. The gel-dye mix was vortexed well and spun at 13000g for 10 minutes at room temperature. A new RNA

6000 Nano chip was placed on the chip priming station. Nine microliters of gel-dye mix was pipetted into the well marked Ⓖ. Plunger was positioned at 1 ml and then the priming station was closed. Plunger was pressed until it was held by the clip. After exactly 30 seconds, clip was released. Five seconds later, plunger was slowly pull back to 1 ml position. Priming station was opened, 9 µl of gel-dye mix was pipetted into the wells marked G. Rest of the gel-dye mix was discarded. Five microliters of RNA 6000 Nano was added to rest of the wells. All the samples were heated to 70°C for 2 minutes to denature the secondary structure of RNA. One microliter of the RNA samples were added to the sample wells, 1 µl of RNA 6000 Nano Marker was added in the sample well without RNA sample inside. One microliter prepared ladder was added into the ladder well. The chip was placed horizontally in the adapter of the IKA vortexer and vortexed for 1 minutes at 2400 pm. Chip was run within 5 minutes. The RNA quality was identified by the Eletrophresis File Run Summary.

2.5.3 cDNA synthesis

GE® First Strand Synthesis Kit was used for cDNA synthesis. Around 1.5 µg of RNA was taken for each sample and the total volume was adjusted to 8 µl in total. The RNA was heated to 65°C for 10 minutes, then chilled on ice. Then a master mix was prepared, a single reaction contains 1 µl primer, 1 µl DTT solution and 5 µl first-strand reaction mix. The master mix was added to the denatured RNA solution, and incubated at 37°C for 1 hour. The synthesized cDNA was aliquoted into 7 tubes and stored in -80°C for long-term storage.

2.5.4 QPCR

Platinum® SYBR® Green qPCR Kit was used for qPCR analysis. cDNA was removed from the -80°C and placed on ice. Individual cDNA samples were diluted to 90 µl with RNase-free water. A master mix was prepared: single reactions contain 6.25 µl of Platinum SYBR Green qPCR SuperMix UDG, 0.25 µl ROX Reference Dye, 0.5 µl pure water. Then, primer for each candidate gene was added into the aliquot of master mix (each gene required both Forward and Reverse primer, each well needed 0.5 µl each primer and 7 µl of master mix). A new 96-well plate was placed on the cold block. cDNA (4.75 µl) was added into each well followed by 7.5 µl of the master mix with primers. Reactions were performed in triplicate for each cDNA sample. ROX and SYBR green fluorescence was measured during the PCR run. The qPCR program had 4 segments: in the 1st segment, the temperature was set to 50°C for 2 minutes, then set to 95°C for 2 minutes: in the 2nd segment, 40 cycles were repeated, temperature was set to 95°C for 15 seconds, followed by 60°C for 30 seconds; in the 3rd segment, temperature was set at 95°C for 1 minute, then 60°C for 30 seconds, later 95°C for 15 seconds; in the 4th segment, temperature was set to 25°C for 30 seconds.

2.6 Data analysis

2.6.1 Myofibre number and cross-section area (CSA)

Care was taken to ensure that for different muscle samples, similar cross-sectional areas were analysed. For each analysed section, 5 images under 400X magnification were randomly selected. Photographic images represented an area of 220 µm by 166 µm. Myofibre numbers were counted for the whole image (myofibres on the borders were only counted on the top and left side of the photo). The cross-section area of the

myofibres was measured by Image J manually, each complete myofibre in the photo was measured and recorded, and the average CSA and number was calculated for each image. Student T-test was used to compare male group and female group data.

2.6.2 PAX7 positive cells number

Five images were generated for each immunostained sample: five 30X30 μm boxes were chosen randomly from within muscle fascicles, and both PAX7-positive cell numbers and total nuclei numbers were estimated. The ratio of PAX7 positive cells to total nuclei in each area was calculated, and then the average for each image calculated. T tests were used to compare the ratio of PAX7 positive cells and total nuclei between male group and female group.

2.6.3 Analysis of gene expression

For routine QPCR the fluorescence threshold was set at 200 dR, and individual Cts calculated. The average Ct for triplicate groups was calculated and corrected against the average Ct for HMBS (expression of this gene had previously been demonstrated to be the most stable in the developing gonad: compared to beta-actin and GAPDH). DCt was calculated as Ct (HMBS) minus Ct (GOI). The DDCt was calculated as the DCt of a single male sample minus the DCt of individual sample. Relative expression level was calculated as (2 to the power -DDCt). Average of male group and female group was calculated, and T test was carried out to compare the difference between male and female groups.

3.0 RESULTS

3.1 Morphology of male and female chick embryo breast muscle

3.1.1 Analysis of myofibre number and cross sectional area

We have previously demonstrated that the male chick embryo has a greater mass of breast muscle than the female chick embryo, at E12.5. In order to characterise this male:female difference, we carried out a series of histological analyses on breast muscle from male and female embryos at E 12.5 of development. Fertilised eggs were incubated until E12.5 and then breast muscle dissected from a minimum of five male embryos and five female embryos.

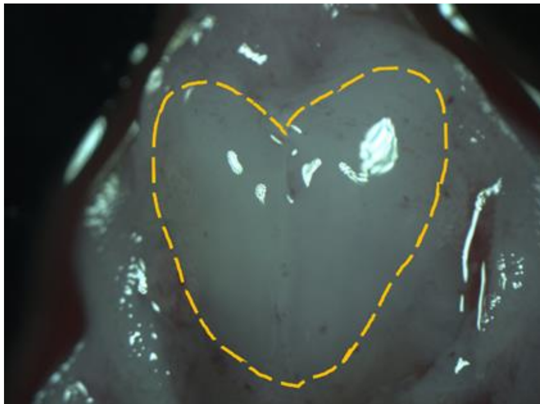

Figure 3-1. Following removal of the skin, breast muscle was dissected from the region indicated by the yellow dotted line.

Embryos were initially sexed by visual examination of the gonads and then sexing was confirmed by Invader assay (Clinton et al, 2016). The Invader assay is a FRET-based enzymatic assay that generates distinct fluorescent signals from complementary probes to two separate DNA sequences. One of the sequences is a repeat sequence common to both males and females while the other sequence is female-specific. In the presence of these target DNA sequences, the complementary FRET probes are cleaved to release a fluorescent ROX signal (common sequence) or a fluorescent FAM signal (female

specific). Female samples generate both FAM and ROX signals while male samples generate only the ROX signal (Figure 3-2).

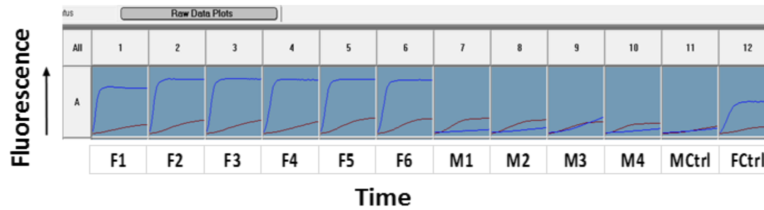

Figure 3-2. Invader assay: screen shot of fluorescence profiles over time. Signal from female-specific target (blue) accumulates rapidly only in female samples (F1-F6), while the signal from the common sequence (red) accumulates in both male and female samples (M=male; F=female: Control=Ctrl)).

Fixed and embedded muscle samples were sectioned by cryostat and the resulting sections immunostained for Laminin, which clearly delineates the boundary of individual myofibres (Figure 3-3). Sections from similar regions of individual muscle samples were selected for myofibre analysis. Five randomly selected images were captured from sections of individual muscles. Myofibre numbers were counted and mean myofibre density calculated for five male

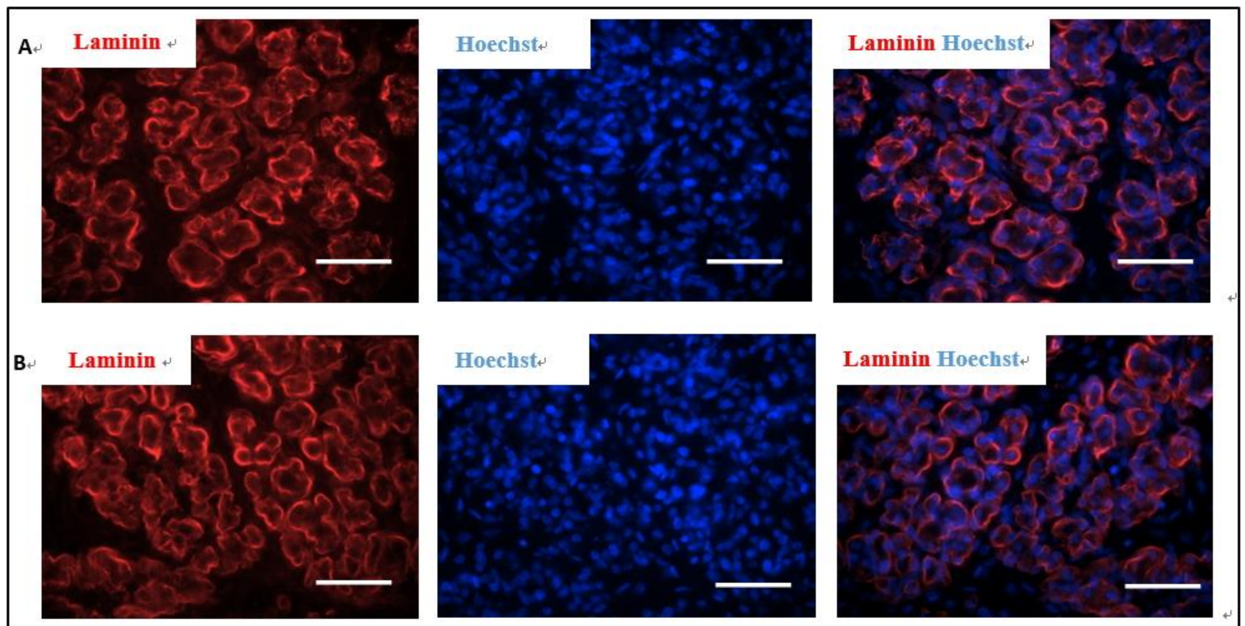

Figure 3-3. Sections through E12.5 male and female embryonic breast muscle. Male and female sections were immunostained together using an antibody against Laminin, and counterstained with Hoechst. Laminin staining clearly delineates the border of individual myobires and Hoechst stains cell nuclei. Irregular outline of myofibres suggests that myotubes are undergoing fusion. Size bar = 50 µm

and five female samples. Figure 3-4 shows the average myofibre number for individual muscle samples.

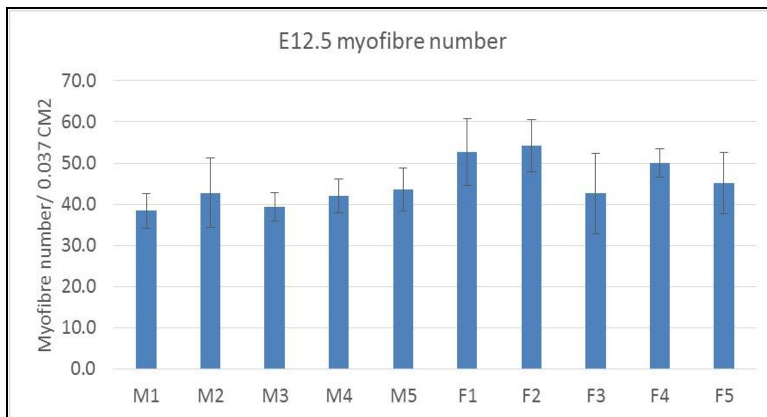

Figure 3-4 Number of myofibres present in defined area of male and female breast muscle of E12.5 chick embryos. Histogram bars represent the average number of myofibres ± 1.S.D in individual male and female embryos

The mean myofibre number in five male muscle samples and five female muscle samples is shown in Figure 3-5.

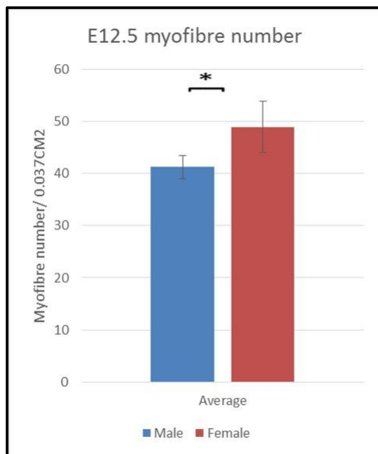

Figure 3-5. Mean number of myofibres in a defined area of male and female breast muscle of E12.5 chick embryos. Data represent mean ± 1.S.D *P<0.05

This shows that myofibre density is significantly higher in female breast muscle than male breast muscle at E12.5 (*P<0.05). Image J software was used to manually trace the outline of all myofibres in each image and to calculate the cross sectional area (CSA) of individual fibres. The average CSA in individual muscle samples is shown in Figure 3-6,

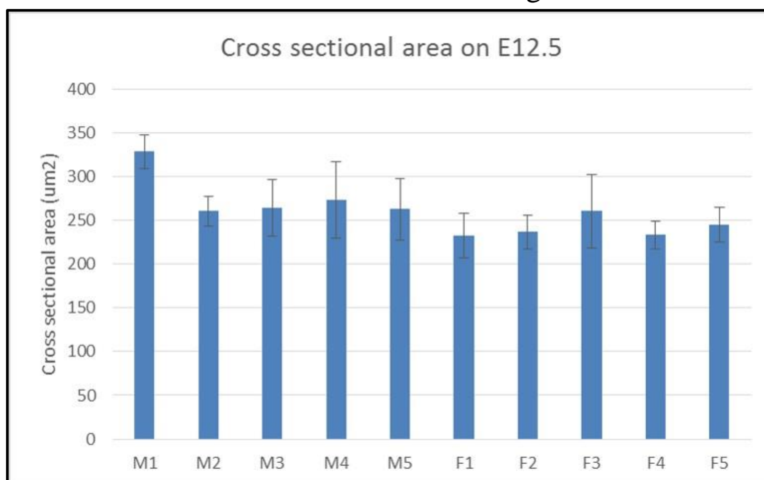

Figure 3-6. Average cross sectional area of myofibres in individual muscle samples ± 1.S.D.

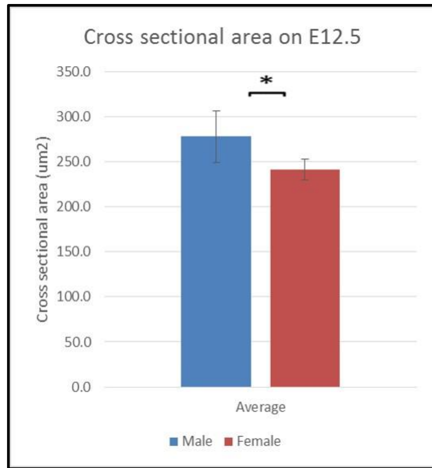

Figure 3-7. Mean cross sectional area in defined area of male and female breast muscle from E 12.5 chick embryos. Data are mean ± 1.S.D *P<0.05

and the mean CSA in combined male and combined female samples is shown in Figure 3-7. This shows that the mean CSA in male muscle samples is significantly greater (*P<0.05) than the mean CSA in female muscle samples, at E12.5 of development. Overall, it appears that male breast muscle has a smaller number of larger myofibres than female breast muscle at E12.5.

3.1.2 Pax7 positive cells in E12.5 chicken breast muscle

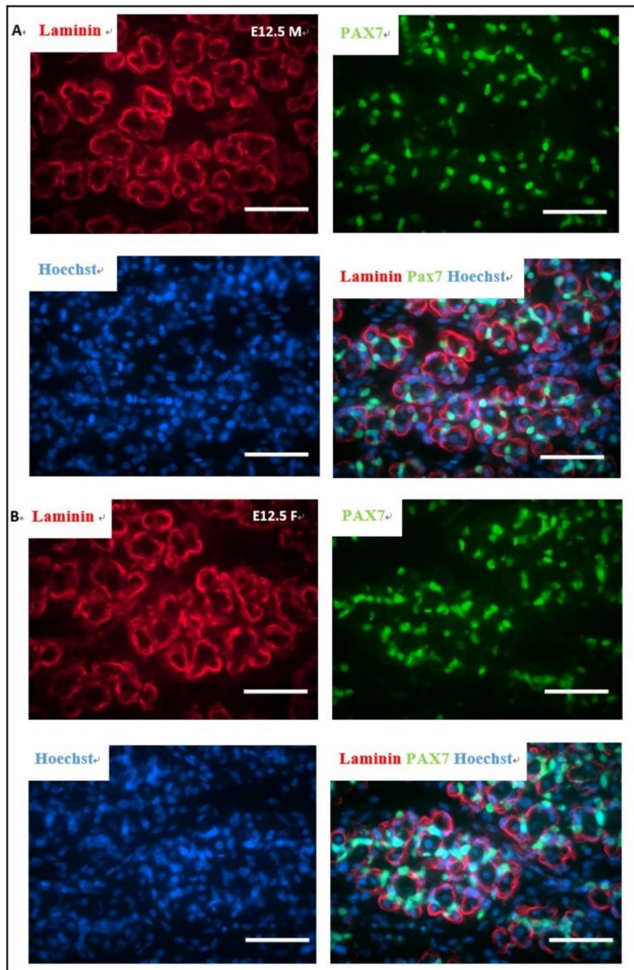

Figure 3-8. Pax7, laminin and Hoechst staining in E12.5 male (A) and female (B) chicken breast muscle. Pax7+ve cells are found inside and outside myofibres.

Satellite cells contribute to increases in muscle mass in adult vertebrates and we wished to evaluate similar cell types in the embryo. Pax7 is a reliable marker of satellite cells in adult muscle (Oustanina 2004) and Pax7 is reported to be expressed in a cell type described as a fetal myoblast in embryonic muscle (Kassar-

Duchossoy et al., 2005; Messina and Cossu, 2009). During secondary myogenesis, Pax7 positive cells differentiate and fuse with the myofibres. In order to compare the ratio of Pax7 + cells to total nuclei, sections of E12.5 male and female muscle were stained with antibody against Pax7. Figure 3-8 shows the staining pattern of both laminin and Pax7. Pax7 staining is found in the nucleus, but not all the nuclei are Pax7 positive. The fibrous connective tissue in muscle can be stained using a fluorescent conjugated lectin, wheat germ agglutinin (WGA) as shown in Figure 3-9. It is clear that Pax7 staining is restricted to the muscle fascicle and does not co-

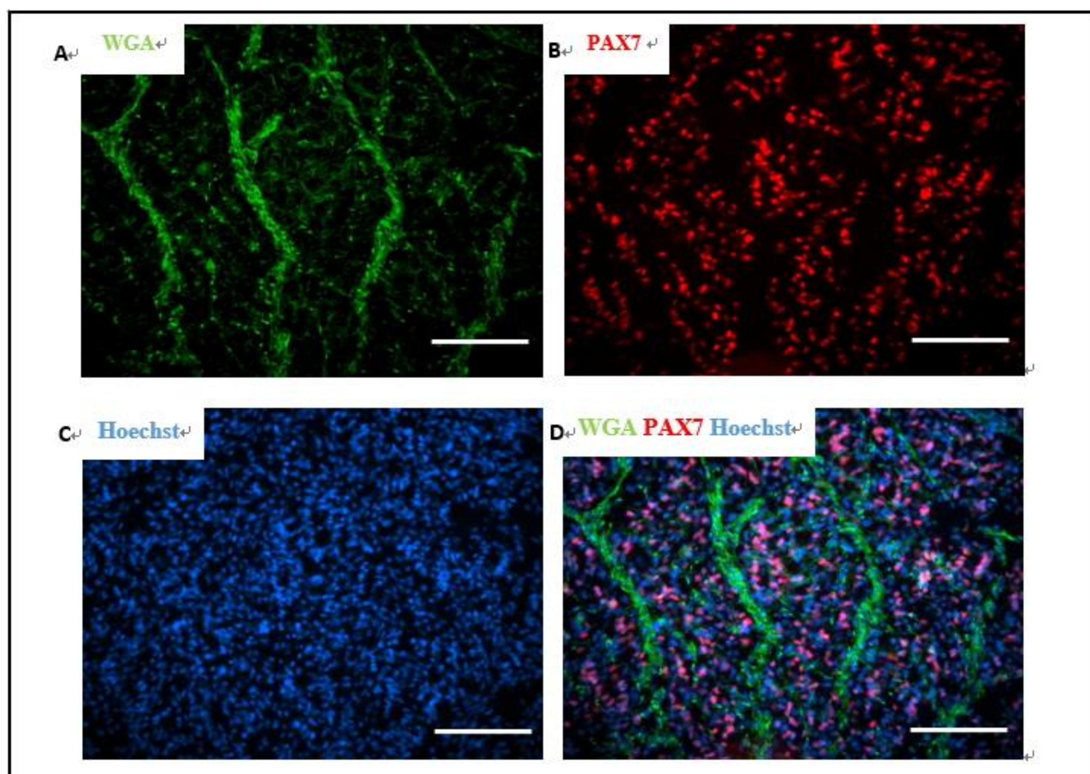

Figure 3-9. Staining pattern of WGA and Pax7 in E12.5 chicken. Pax7 positive cells are only found in the muscle fascicles rather than the connective tissue between fascicles. Bar= 100 µm.

localise with WGA staining. In order to calculate the Pax7=ve/-ve ratios, only nuclei located within the muscle fascicles were counted. Figure 3-10 shows the ratio of Pax7+ve cells to total nuclei within individual muscles. The mean Pax7+ve ratio in male and female muscle is shown in Figure 3-11 , and shows that the Pax7+ve/-ve ratio, in E12.5 chick embryos, is significantly higher in female breast muscle than in male breast muscle (**P<0.01).

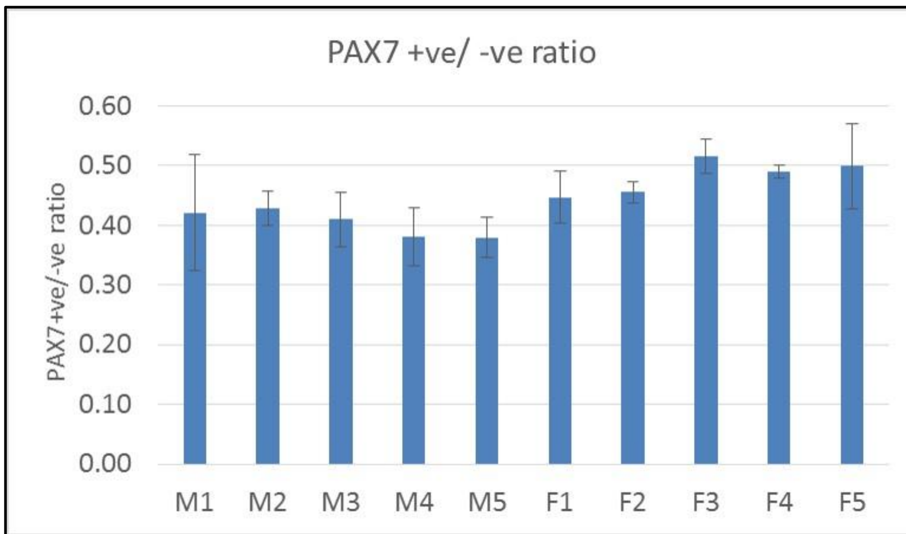

Figure 3-10. Ratio of Pax7 positive cells to total nuclei in fascicles of individual muscle samples (F=female: M=male). Histogram bars represent average ratio of PAX7 +ve/-ve ± 1.S.D

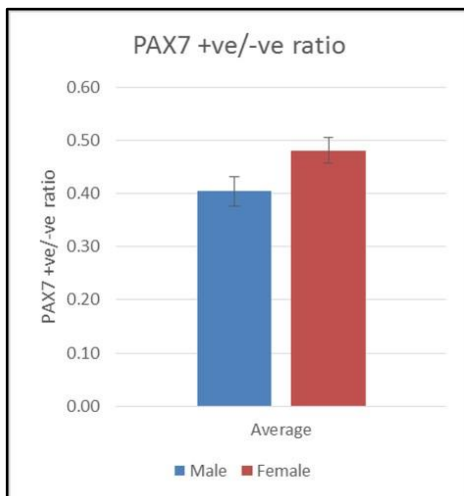

Figure 3-11. Mean Pax7+ve/-ve ratio in male and female breast muscle of E12.5 chick embryos. Female muscle has a significantly higher Pax7 +ve ratio than male muscle (**P<0.01). Data are mean ± 1.S.D

3.1.3 Fascicle formation during E11.5 - E13.5

In order to gain a greater understanding of the dynamics of muscle development around this period of embryogenesis, we also examined gross muscle morphology at E11.5 and E13.5. Once again, breast muscle was collected from five male and five female embryos and sectioned and stained. Figure 3-12 illustrates typical examples of male and female muscle sections from E11.5, E12.5 and E13.5 embryos, immunostained for laminin and counterstained with Hoechst. At E11.5 there is no

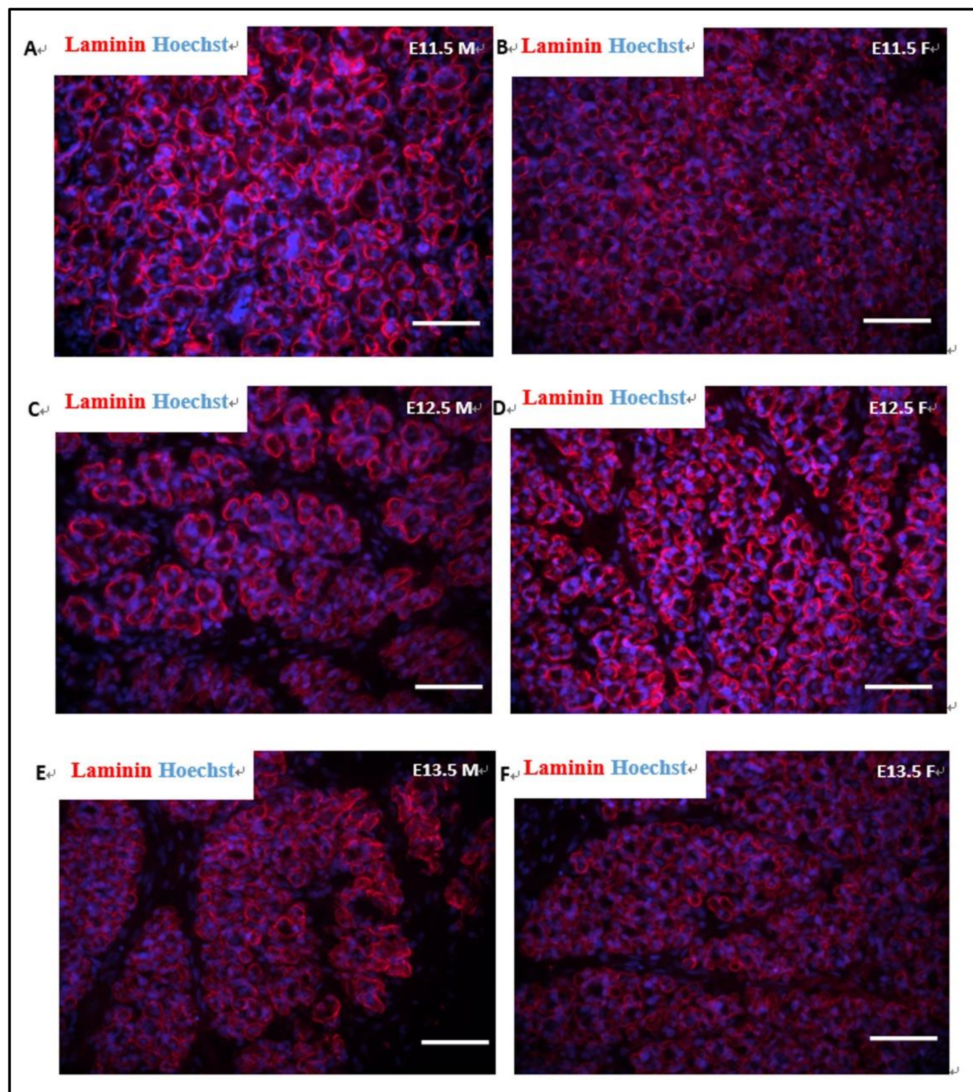

Figure 3-12. Sections of E11.5 (A,B), E12.5 (C,D) and E13.5 (E,F) chicken breast muscle were stained with antibody against Laminine (arrow). Fascicles gradually form over this period and myofibre fusion is observed. (M=male: F=female) Bar= 50 µm.

obvious fascicle formation and the myofibre cross sections are generally circular. At E12.5, muscle fascicle formation has obviously begun and myotubes appear to be fusing together and generating an irregular myofibre cross sectional outline. By E13.5, fascicle formation is clear with substantial connective tissue regions. At this stage the myofibre cross section appears diminished compared to that seen at E12.5. It appears secondary fusion and fascicle formation is initiated around E12.5. The fusion pattern of E12.5 breast myofibres seems to be the secondary myotubes surrounding and fusing with the primary myotubes.

3.1.4 Chimeric muscle investigation

Although assessing morphology of muscle from the analysis of numbers of individual male and female samples, does generate robust reliable data – extreme care must be taken with this form of approach. For instance, it is important that very similar regions of muscle prepared in exactly the same manner and sectioned in the same plane, are compared. This is by no means trival to ensure and the inherent variation can be compounded by slight differences in processing individual muscles and processing individual sections. With this approach, potential problems with reproducibility can only be overcome by assessing numbers of samples. For these reasons, we decided to investigate the potential of an alternative approach – the generation of mixed-sex chimeras that possessed breast muscle that contained regions of male tissue and regions of female tissue. By analysing male and female regions of the same chimera, we can exclude the possibility that tissue processing or section processing influences our conclusions – as our assessment will be carried out on a single (mixed-sex) tissue and measurements will be made on single sections. As the tissue will originate from a single individual, we could also exclude the

possibility that any male:female differences observed were due to the influence of hormones or growth factors. To generate such chimeras, a portion of lateral plate mesoderm (LPM) is transplanted from a very early chick embryo (E2.0) and used to replace the identical region in a second E2.0 embryo. In order to track the transplanted tissue, LPM from a Green Fluorescent Protein-expressing (GFP) embryo is transplanted into a wild-type embryo. The transplant surgery is performed through a small window cut in the eggshell, which is then sealed and the egg reincubated for a further 10 days. During this period, the transplanted cells will proliferate, differentiate and contribute to a number of tissues on the transplanted side of the embryo – including breast muscle. Figure 3-13 shows a typical example of such a chimeric embryo at E12.5 of development, with the breast muscle exposed and illuminated by UV light. It is clear that a significant proportion of the breast muscle on the left side of this embryo is comprised of GFP-expressing cells derived from the transplanted LPM, while the breast muscle on the right-hand side is composed entirely from wild-type cells. In this instance the GFP-expressing cells were derived from a male 'donor' embryo and were transplanted into a female 'host' embryo. Ideally, the combined left and right breast muscle of such a chimera should have been treated as a single tissue and sectioned across the cartilage sternum –

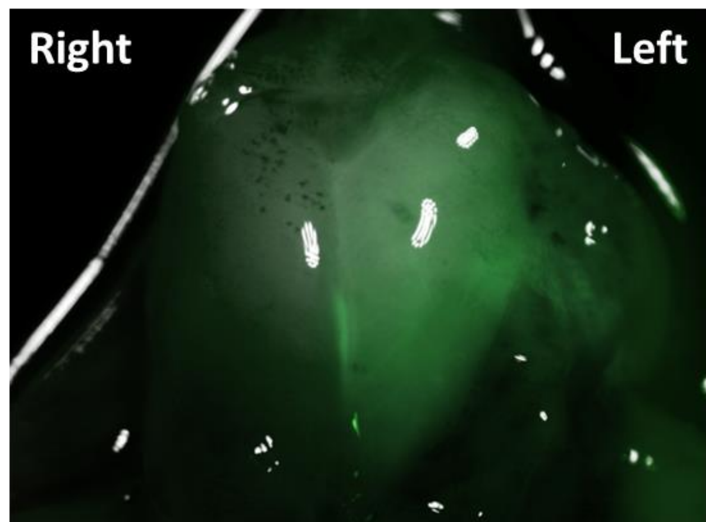

Figure 3-13. Breast muscle from chimeric embryo (D Zhao). GFP donor tissue from a male chicken embryo, transplanted into a wild type female host.

generating a single section where one portion was composed entirely of female cells and the other portion was comprised mainly from male cells. Unfortunately, left and right breast muscles were processed separately. Nevertheless, the standard procedures did generate a series of sections that ranged from muscle tissue almost entirely composed of male cells, to muscle tissue with an equal mix of male and female cells to muscle tissue that was composed mainly of female cells. A variey of illustrative sections are shown in Figure 3-14 (chimeric embryos were generated by D Zhao). In A and B panels the majority of the cells are male, while in C and D panels, the majority of the cells are female. Although these are only single sections from a single chimera, we carried out an analysis of myofibre number and myofibre cross-sectional area. In general, the data is in agreement with our analyses of individual male and female muscle samples: male-rich tissue was comprised of a smaller number of large fibres, while female-rich tissue contained a greater number of smaller fibres (Table 1). While obviously simply preliminary data, these findings are encouraging for the use of chimeric embryos as an experimental approach.

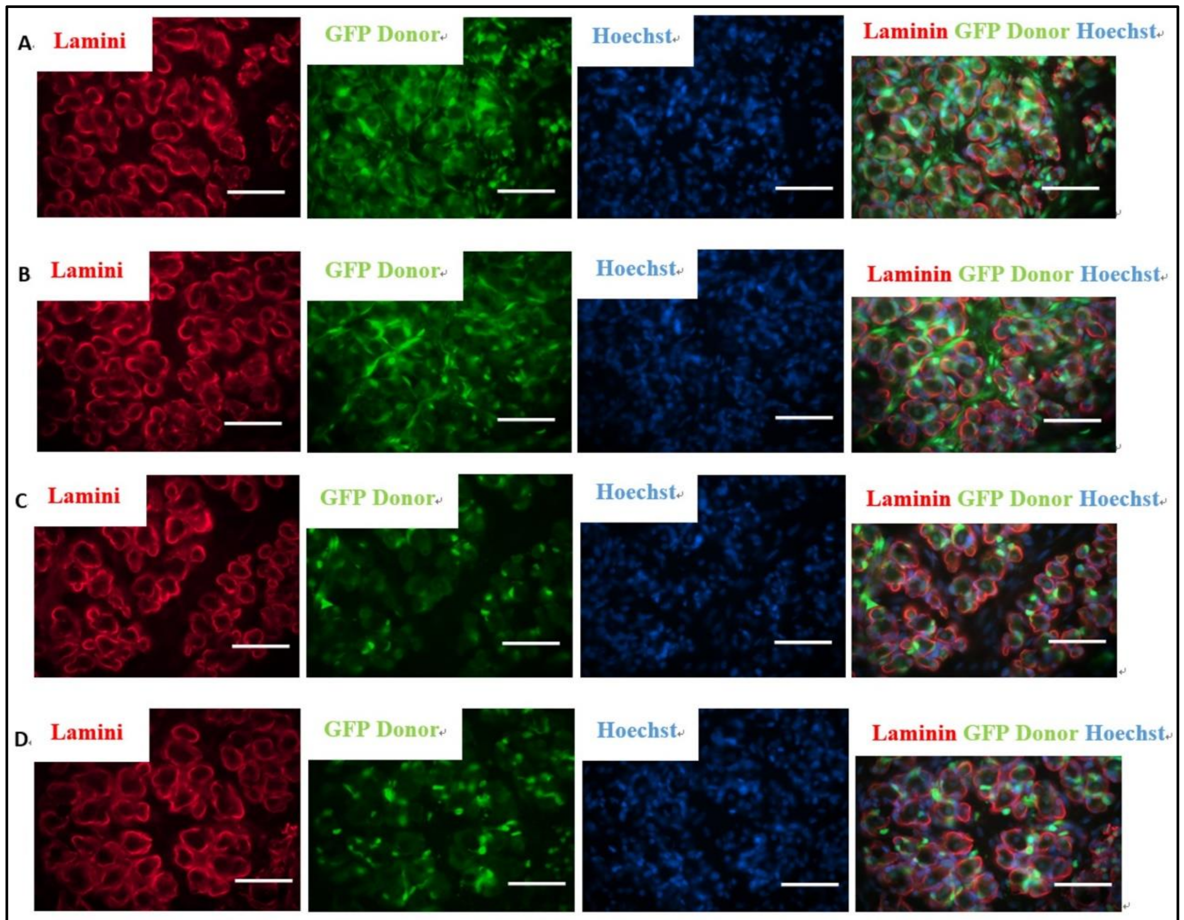

Figure 3-14. Immunostaining of muscle sections from chimeric embryo. A and B show regions with high GFP-donor content and C and D show regions with low GFP-donor content. GFP donor is male and wild-type host is female. Bar = 50 µm.

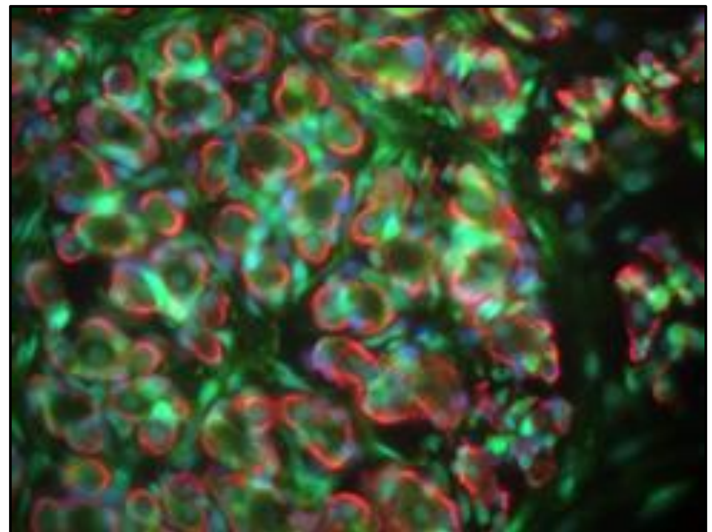

Figure 3-14 (2). Enlarged image of 3-14 panel A

Picture	MFN	CSA(μm²)
A	37	6430.151
B	38	6689.906
C	45	5564.454
D	44	6606.4

Table 1 Myofibre number (MFN) and cross sectional area (CSA) derived from section illustrated in Figure 3-14.

3.2 Gene expression in developing male and female chick embryo breast muscle

Previous findings from our laboratory (Zhao et al, 2010) have established that sexual dimorphisms in birds are largely due to inherent cellular sex identity – i.e. cell autonomous factors. As part of an RNA-seq analysis investigating the underlying molecular mechanisms, a number of genes that displayed sexually dimorphic expression in embryonic muscle were identified. One of these genes, muscle-specific kinase (MuSK) is only expressed in skeletal muscle. MuSK is reported to be the key factor in neuromuscular junction (NMJ) formation (DeChiara et al., 1996; Okada K et al., 2006). MuSK is expressed within skeletal muscle cells and facilitates the accumulation of the elements required to form the post-synaptic portion of the NMJ – if MuSK is depleted, no NMJs form. It is obvious from the literature (Pellegrino et al, 1963; Sartori et al., 2013) that there is a close relationship between innervation and muscle mass – chemical or surgical denervation leads to muscle atrophy. This

raised the possibility that the higher levels of MuSK expression in male muscle lead to the formation of increased numbers of NMJs and the survival of a greater number of myofibres in male muscle. To further explore this possibility, we elected to measure expression of MuSK and of a number of genes associated with neuronal formation/function, in male and female breast muscle at a number of stages during development – E8.5, E10.5, E12.5, E14.5 and E16.5. The Real-time quantitative PCR (qPCR) assays used in these analyses had previously been optimised in our laboratory and conformed to the required efficiency over a 1000-fold dilution range (95%-105% efficiency).

We also attempted to use bungaratoxin staining to quantify NMJs in male and female muscle, however this approach proved to be impractical. During development, the embryo matures in an anterior to posterior fashion – so, at particular stages, anterior portions of the breast muscle are at a more advanced stage of myogenesis than posterior portions. Because of this, it proved impractical to reproducibly generate the *longitudinal* sections required to reliably estimate NMJ numbers.

3.2.1 MuSK expression

MuSK expression level was measured in the breast muscle of both male and female chick embryos at different stages between E8.5 and E16.5. Figure 3-15 shows the expression in female muscle relative to male muscle at each individual stage. It is clear that MuSK is expressed at significantly higher levels (~2-fold) in male breast muscle than in female breast muscle at all stages of development tested.

Figure 3-16 shows the expression of MuSK in chick embryo breast muscle at different stages of embryonic development, relative to a single male individual at

E8.5. It is clear that MuSK expression levels increase significantly in both male and female breast muscle over the period of secondary myogenesis.

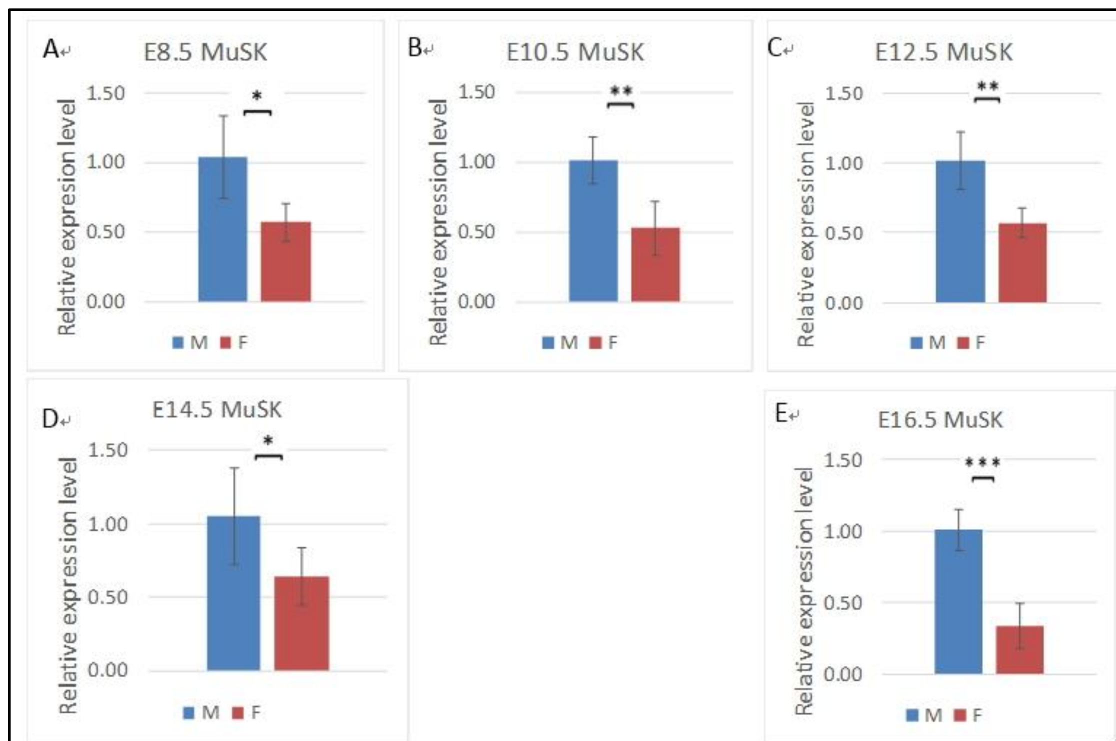

Figure 3-15. Expression of MuSK in female breast muscle relative to male breast muscle at different developmental timepoints (A= E8.5, B= E10.5, C= E12.5, D= E14.5 and E= E16.5). MuSK expression level is significantly higher in male chicken breast muscle than female chicken breast muscle on E8.5 (*P<0.05), E10.5 (**P<0.01), E12.5 (**P<0.01), E14.5 (*P<0.05) and E16.5 (***P<0.001).

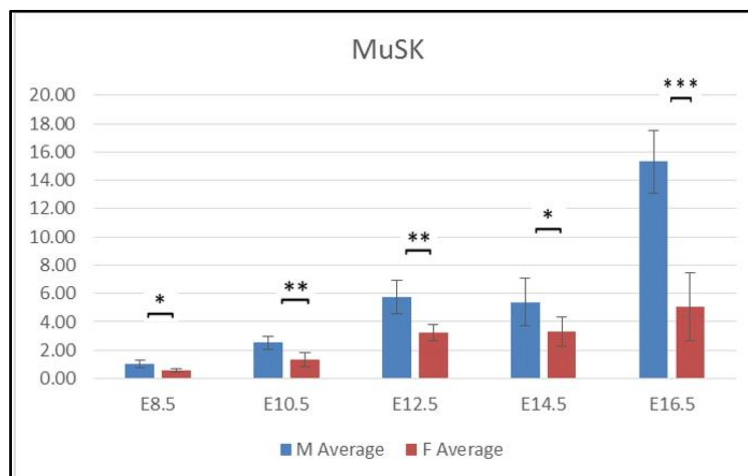

Figure 3-16. Change in MuSK expression level during muscle development. The developmental period covered, encompasses the later stages of primary myogenesis and secondary myogenesis. It is clear that MuSK levels increase dramatically during myogenesis (10-15 fold). [M=male: F=female]

3.2.2 Expression levels of selected genes associated with neuronal formation/function in male and female breast muscle

Our RNA-seq analysis identified additional genes that displayed sexually dimorphic expression in chick embryo breast muscle and a number of these genes were selected for characterisation during muscle development, because of an association with neuronal development or function. The genes selected for further analyses were Synuclein Alpha Interacting Protein (SNCAIP), Dimethylglycine dehydrogenase (DMGDH) and Ciliary neurotrophic factor receptor (CNTFR). The SNCAIP gene encodes synphilin-1 protein which interacts with alpha-synuclein in neuronal tissue and is located in the presynaptic terminals of NMJs (Ribeiro et al., 2002). DMGDH encodes an enzyme involved in the catabolism of choline, catalyzing the oxidative demethylation of dimethylglycine (DMG) to form sarcosine (Binzak et al., 2001). Although widely expressed, mutations in this gene can lead to sarcosinemia which in turn leads to a number of muscular and neuronal disorders. The CNTFR gene encodes a member of the type 1 cytokine receptor family. The encoded protein is the ligand-specific component of a tripartite receptor for ciliary neurotrophic factor, which plays a critical role in neuronal cell survival, differentiation and gene expression. The CNTF protein is a polypeptide hormone whose actions appear to be restricted to the nervous system where it is a potent survival factor for neurons, and where it promotes neurotransmitter synthesis and neurite outgrowth in certain neuronal populations.

SNCAIP expression

SNCAIP expression level was measured in the breast muscle of both male and female chick embryos at different stages between E8.5 and E16.5. Figure 3-17 shows

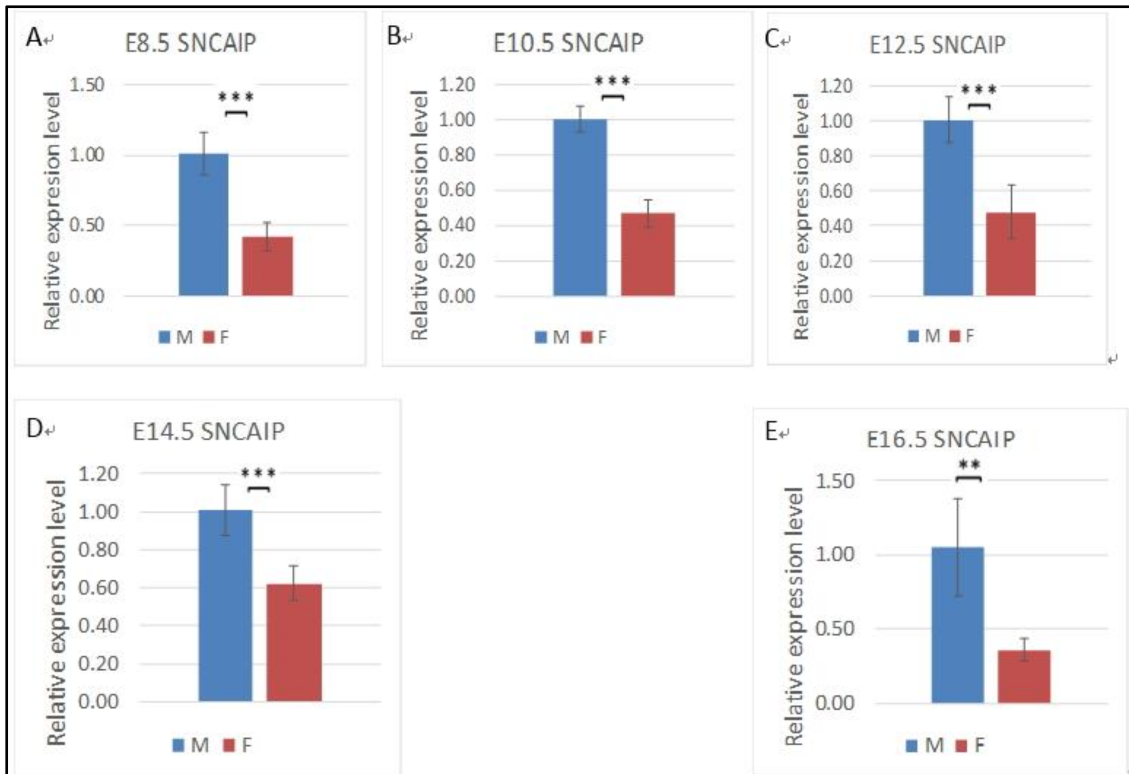

Figure 3-17. Expression of SNCAIP in female breast muscle relative to male breast muscle at different developmental timepoints (A= E8.5, B= E10.5, C= E12.5, D= E14.5 and E= E16.5). SNCAIP expression level is significantly higher in male chicken breast muscle than female chicken breast muscle on E8.5 (***P<0.001), E10.5 (***P<0.001), E12.5 (***P<0.001), E14.5 (***P<0.001) and E16.5 (**P<0.01). [M=male: F=female].

the expression in female muscle relative to male muscle at each individual stage. SNCAIP is expressed at significantly higher levels in male breast muscle than in female breast muscle at all stages of development tested.

Figure 3-18 shows the expression of SNCAIP in chick embryo breast muscle at different stages of embryonic development, relative to a single male individual at E8.5. It is clear that SNCAIP expression levels do not change in male and female breast muscle over the period of secondary myogenesis.

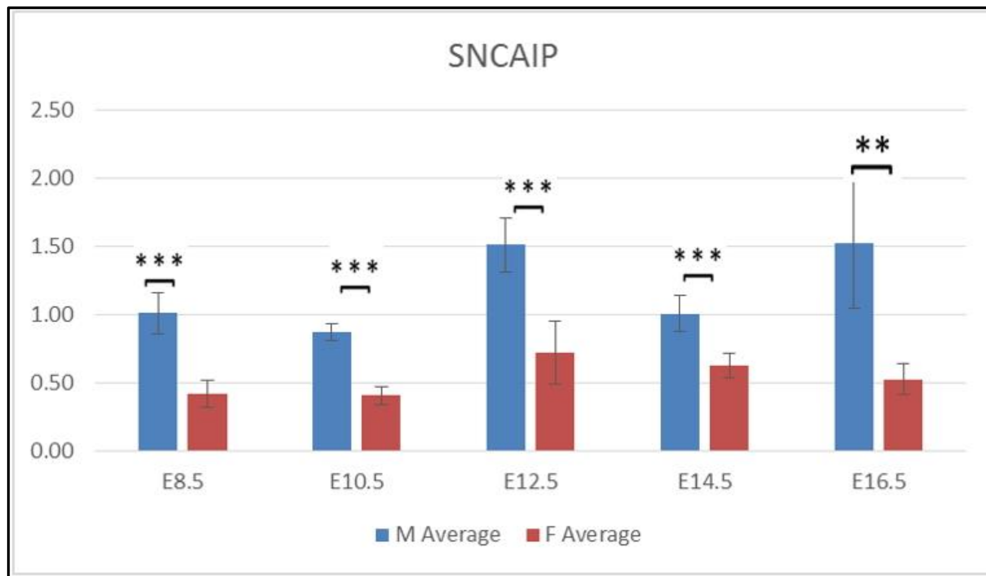

Figure 3-18. Change in SNCAIP expression level during muscle development. The developmental period covered, encompasses the later stages of primary myogenesis and secondary myogenesis. It is clear that SNCAIP expression levels do not change significantly during this period. [M=male: F=female]

DMGDH Expression.

DMGDH expression level was measured in the breast muscle of both male and female chick embryos at different stages between E8.5 and E16.5. Figure 3-19 shows the expression in female muscle relative to male muscle at each individual stage.

DMDGH is expressed at significantly higher levels in male breast muscle than in female breast muscle at all stages of development tested.

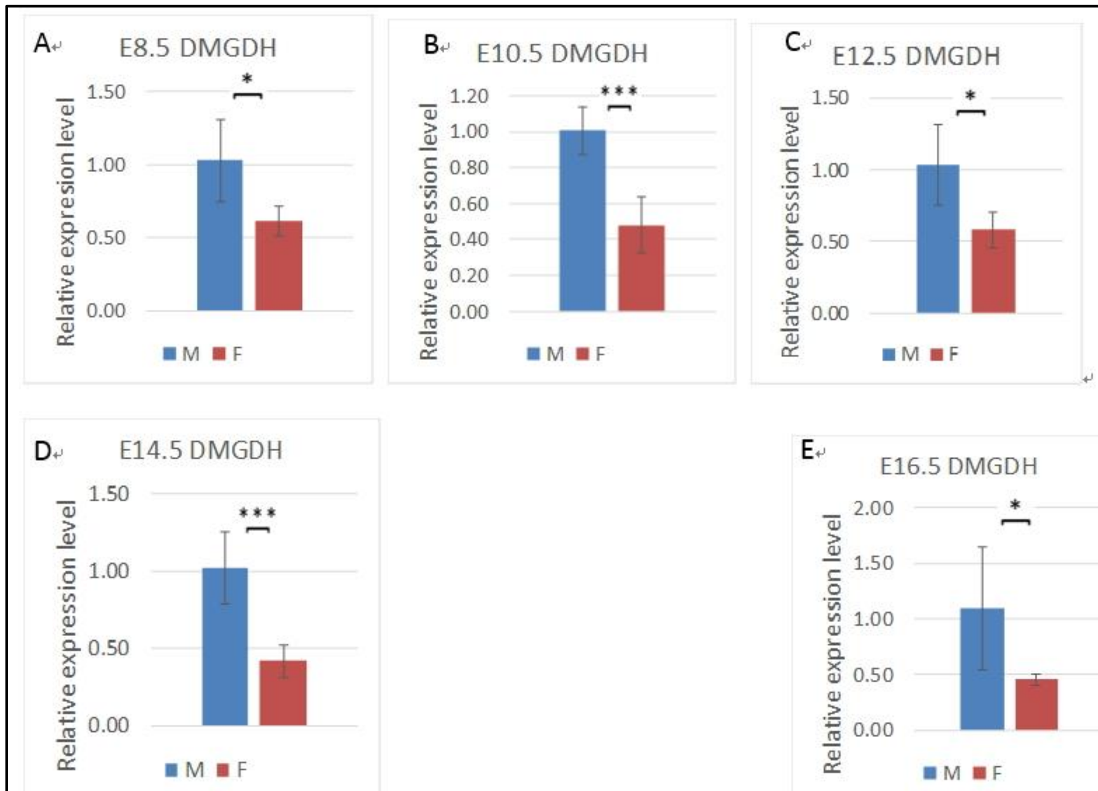

Figure 3-19. Expression of DMGDH in female breast muscle relative to male breast muscle at different developmental timepoints (A= E8.5, B= E10.5, C= E12.5, D= E14.5 and E= E16.5). DMGDH expression level is significantly higher in male chicken breast muscle than female chicken breast muscle on E8.5 (*P<0.05), E10.5 (***P<0.001), E12.5 (*P<0.05), E14.5 (***P<0.001) and E16.5 (*P<0.05). [M=male: F=female].

Figure 3-20 shows the expression of DMGDH in chick embryo breast muscle at different stages of embryonic development, relative to a single male individual at E8.5. It is clear that DMGDH expression levels increase substantially in male and female breast muscle over the period of secondary myogenesis (10-20 fold).

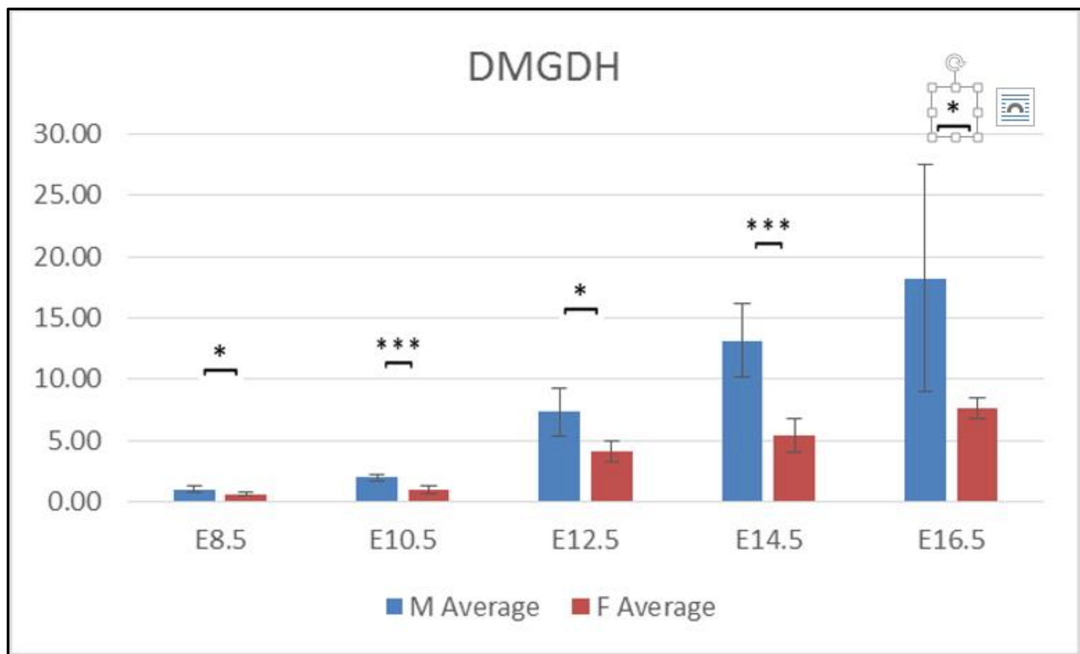

Figure 3-20. Change in DMGDH expression level during muscle development. The developmental period covered, encompasses the later stages of primary myogenesis and secondary myogenesis. It is clear that DMGDH expression levels change signidficantly during this period. [M=male: F=female]

CNTFR Expression

CNTFR expression level was measured in the breast muscle of both male and female chick embryos at different stages between E10.5 and E16.5. Figure 3-21 shows the expression in female muscle relative to male muscle at each individual stage.

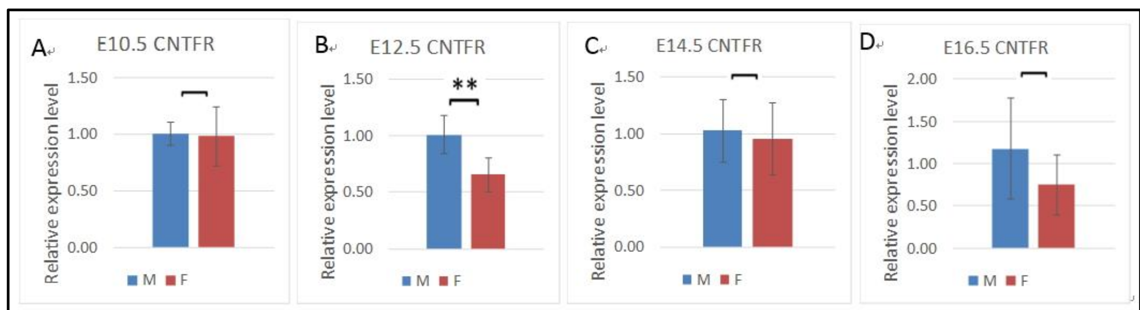

Figure 3-21. Expression of CNTFR in female breast muscle relative to male breast muscle at different developmental timepoints (A= E10.5, B= E12.5, C= E14.5 and D= E16.5). CNTFR expression level is significantly higher in male chicken breast muscle than female chicken breast muscle at E12.5 (**P<0.01).{M=male: F=female].

It is clear that CNTFR is expressed at similar levels in male and female chick embryo breast muscle throughout the majority of the period encompassing secondary myogenesis. Only at E12.5 are male and female levels significantly different, with higher levels of expression of CNTFR seen in male breast muscle than in female breast muscle.

Figure 3-22 shows the expression of CNTFR in chick embryo breast muscle at different stages of embryonic development, relative to a single male individual at E10.5. It is clear that CNTFR shows very little variation in expression level over the period of secondary myogenesis, and is not significantly different between timepoints.

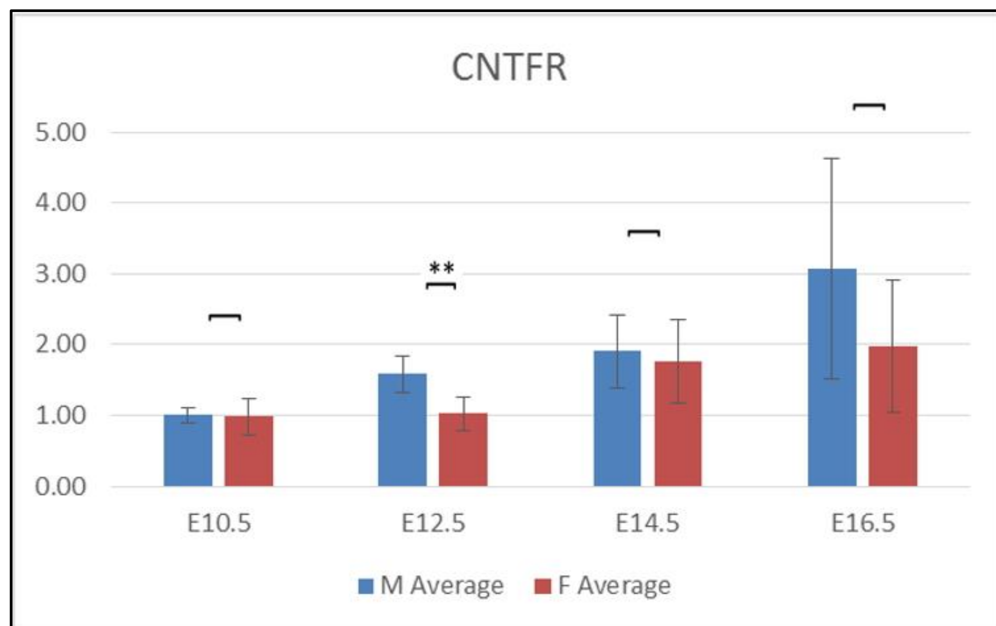

Figure 3-22. Change in CNTFR expression level during muscle development. The developmental period covered encompasses secondary myogenesis. It is clear that CNTFR expression levels do not change significantly during this period. [M=male: F=female]

3.3 BMP signalling in muscle development

While it has been established for some time that BMP signalling plays a role in muscle development (Sartori et al, 2013) it has recently been suggested that MuSK (in conjunction with BMPR) can act as a receptor for BMP signalling (Yilmaz et al. 2016). It was decided to investigate the possibility that the sexually dimorphic expression of MuSK may influence muscle development via a role in BMP signalling rather than via formation of NMJs. To this end, we examined BMP signalling in male and female breast muscle at a variety of levels. Firstly, we compared the expression of various BMPs and BMP Receptor 1 in male and female muscle at E12.5. Secondly, as BMP signalling is transduced via the phosphorylation of SMAD factors, we examined levels of phosphorylated SMAD 1 and 5 (pSMAD 1/5) in male and female muscle. Finally, we measured expression levels in male and female muscle of a factor that during atrophy of muscle, ubiquitinates proteins for degradation, and that is known to be inhibited by BMP signalling (Sartori et al, 2013). This factor is MuSA1 (Muscle ubiquitin ligase of SCF complex in atrophy-1).

3.3.1 Expression of BMPs and receptor

We measured expression of BMP2, BMP4, BMP7 and BMPR1B in breast muscle from five male and five female chick embryos at E12.5 of development (Figure 3-23).

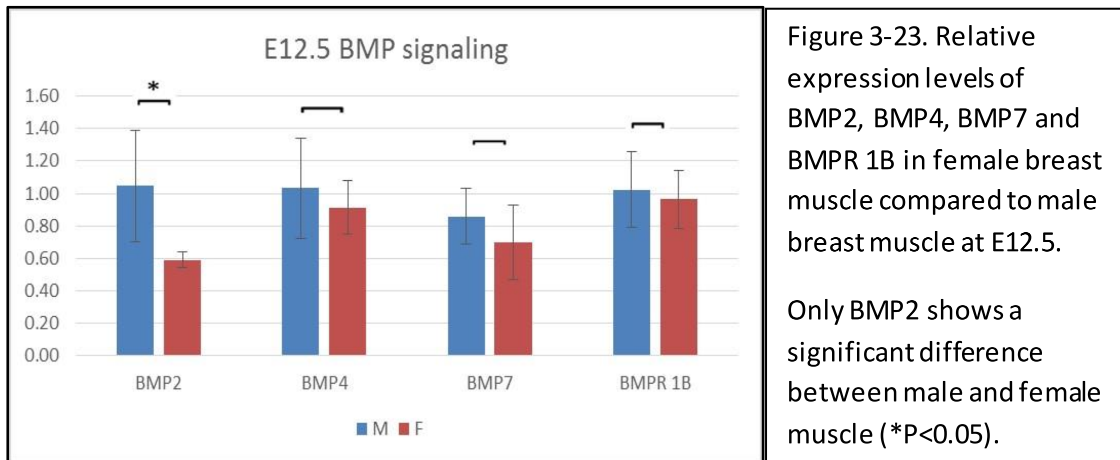

Figure 3-23. Relative expression levels of BMP2, BMP4, BMP7 and BMPR 1B in female breast muscle compared to male breast muscle at E12.5.

Only BMP2 shows a significant difference between male and female muscle (*P<0.05).

Expression levels of BMP4, BMP7 and BMPR1B were similar in male and female breast muscle at E12.5. BMP2 showed sexually dimorphic expression and was expressed at significantly higher levels in male breast muscle than in female breast muscle.

3.3.2 BMP signalling in male and female muscle

We used immunostaining and an antibody against pSMAD 1/5 to examine BMP signalling in male and female breast muscle at E12.5 and E16.5 of development.

Figure 3-24 shows sections of male and female muscle immunostained for pSMAD 1/5 and counterstained with WGA and Hoechst. There is no obvious difference in pSMAD levels in male and female muscle, and it is clear that the most intense pSMAD 1/5 staining localises in the same regions as the WGA staining, which suggests that the BMP signalling is chiefly in the connective fibrous tissue rather than within the myofibres.

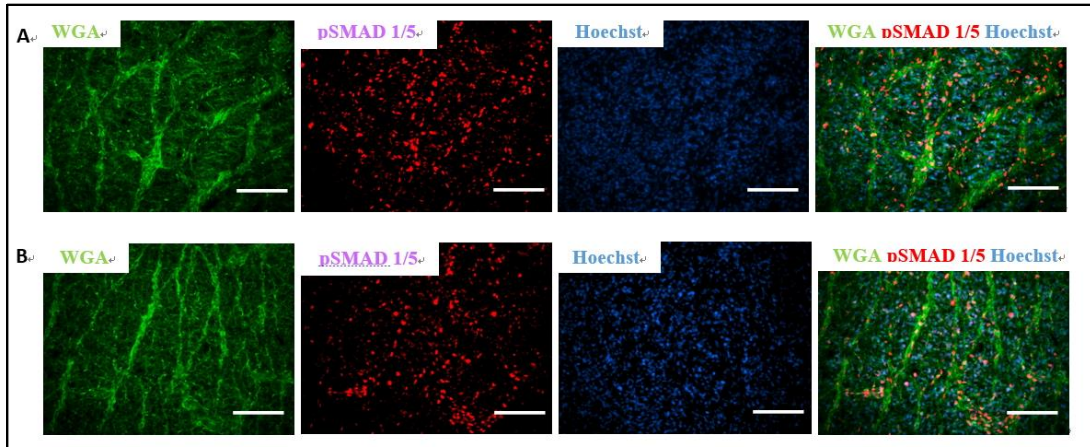

Figure 3-24. BMP signalling in developing muscle. Sections of E12.5 male (A) and female (B) chicken breast muscle stained for pSMAD 1/5 and WGA. Bar=100 µm.

When staining E16.5 male and female muscle sections, we also immunostained for PAX7 to further delineate connective tissue regions from myofibre regions. Figure 3-25 illustrates expression of pSMAD and PAX7 in male and female breast muscle from chick embryos at E16.5 of development.

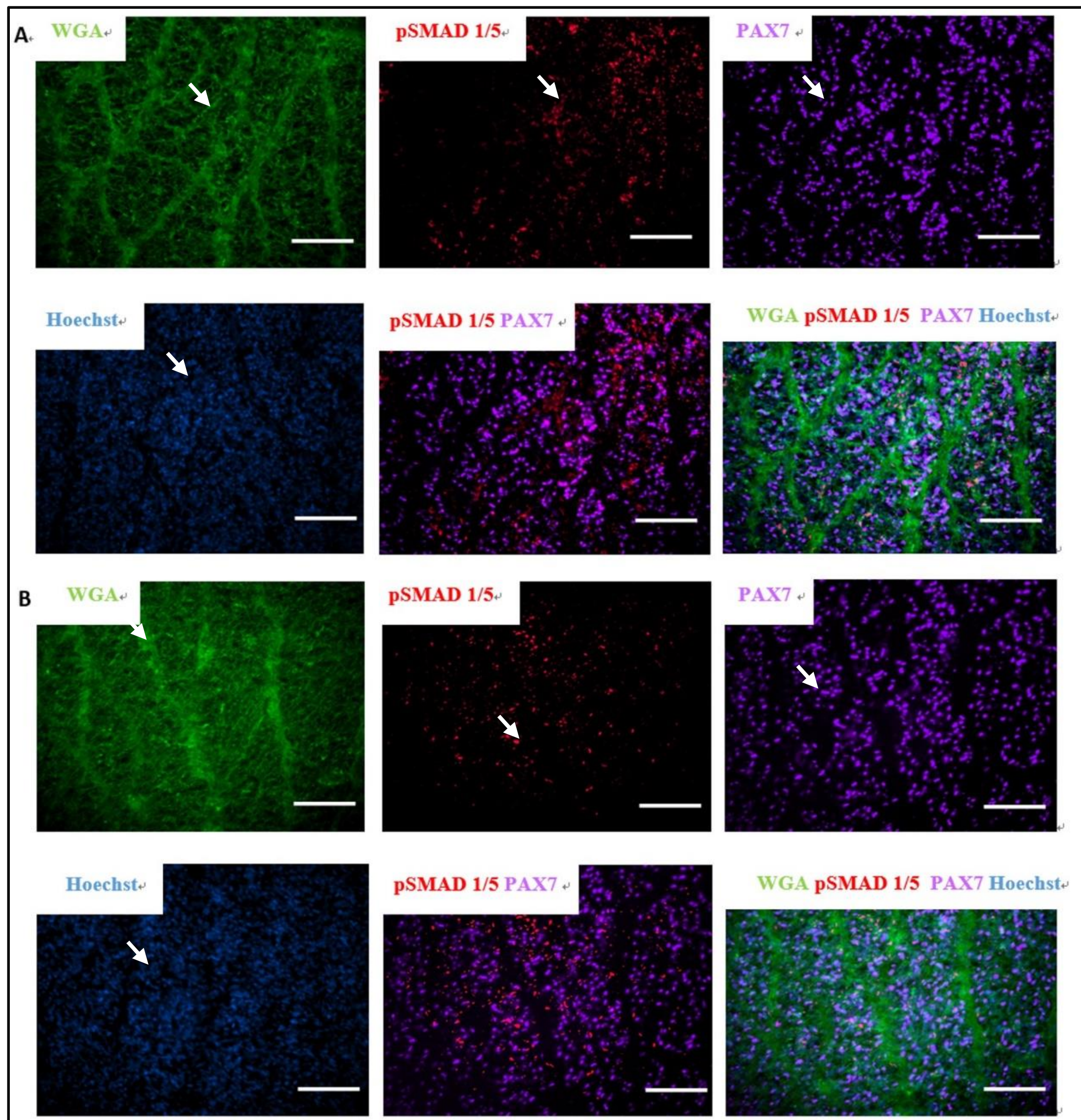

Figure 3-25. BMP signalling in E16.5 male and female breast muscle. Sections of male (A) and female (B) embryonic breast muscle immunostained for pSMAD 1/5 and PAX7 and counterstained with WGA and Hoechst. Figure 3-25-1. Enlarged panel from (A) illustrating that PAX7 and pSMAD 1/5 signals do not overlap. Bar=100 µm

There is no obvious difference in pSMAD signalling between male and female breast muscle and it is clear that the pSMAD signal does not colocalise with the signal obtained using the PAX7 antibody. This again supports the E12.5 findings which

suggest that BMP signalling is enriched in the connective tissue compartment of the muscle, rather than the myofibre compartment.

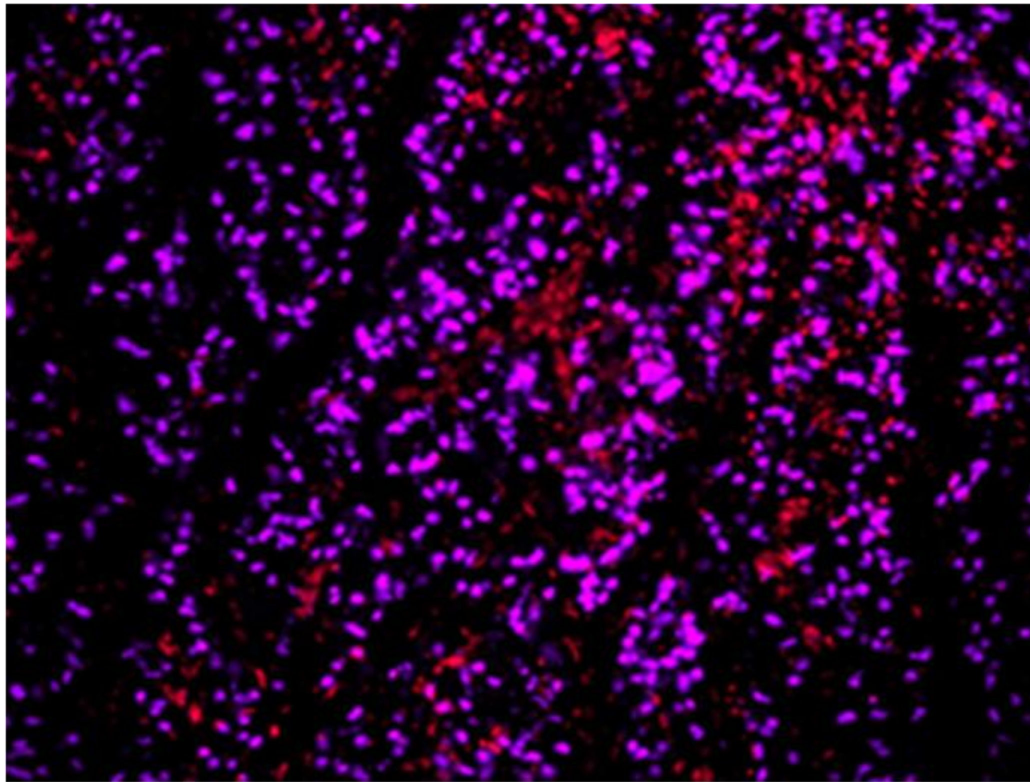

Figure 3-25-1

3.3.3 MuSA1 expression in male and female breast muscle

We measured expression of MuSA1 in the breast muscle of five female and five male embryos at various points in muscle development. Figure 3-26 shows the mean level of MuSA1 expression in male and female breast muscle at E8.5, E10.5, E12.5, E14.5 and E16.5 of development.

MuSA1 expression is significantly higher in male breast muscle than female breast muscle at E8.5 (*P<0.05), whereas MuSA1 levels are equivalent in male and female breast muscle at E10.5. In the latter stages of secondary myogenesis (E12.5, E14.5 and E16.5), MuSA1 levels are significantly higher in female breast muscle than in male breast muscle (***P<0.001, *P<0.05 and *P<0.05 respectively). This may reflect differences in BMP signalling during primary and secondary myogenesis and differences in BMP signalling between male and female muscle, or – if BMP

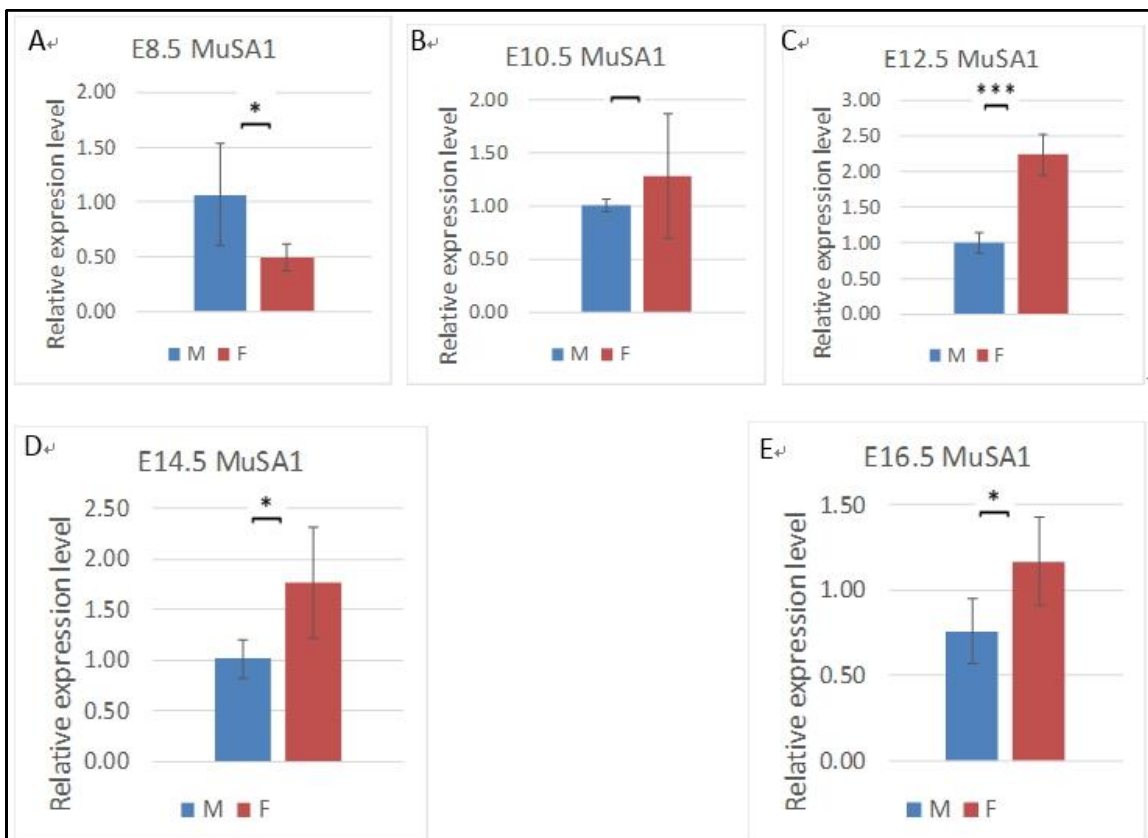

Figure 3-26. Expression of MuSA1 in female breast muscle relative to male breast muscle at different developmental timepoints (A= E8.5, B= E10.5, C= E12.5, D= E14.5 and E= E16.5). {M=male: F=female].

signalling is largely confined to connective tissue, these expression levels may reflect a different distribution of connective tissue in male and female breast muscle.

Figure 3-27 shows relative expression levels of MuSA1 at different stages of development, compared to the expression level of a single male sample from E8.5. This shows that MuSA1 expression levels are highest at E8.5 and E16.5, and in comparison, MuSA1 expression is considerably lower (20 fold) during the key stages of secondary myogenesis (E10.5 –E14.5) – perhaps reflecting increased BMP signalling during this important stage of muscle development?

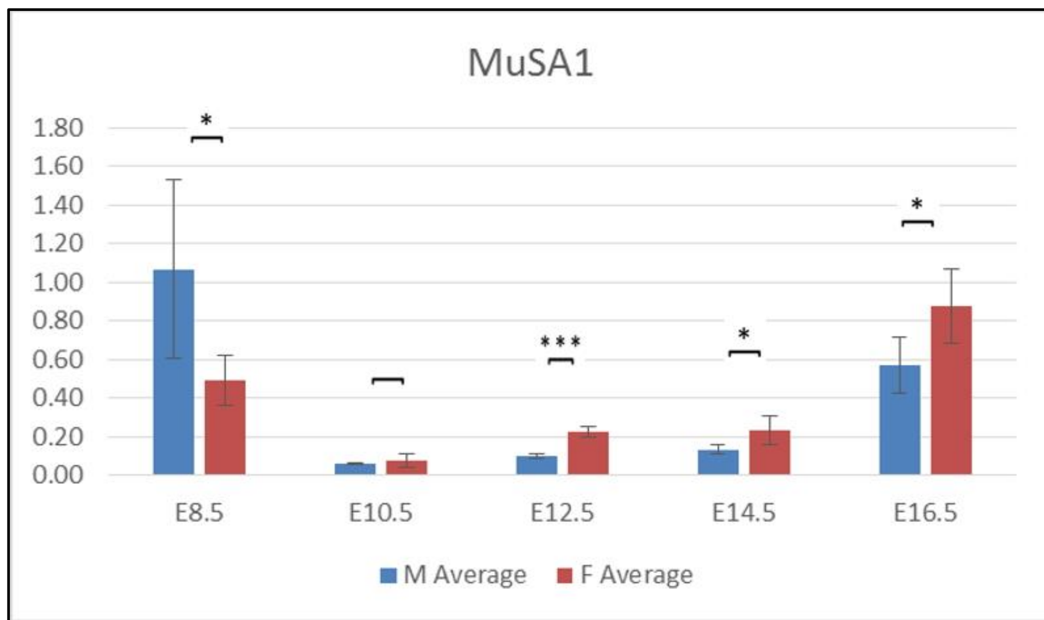

Figure 3-27. Change in MuSA1 expression level during muscle development. The developmental period covered, encompasses the later stages of primary myogenesis and secondary myogenesis. It is clear that MuSA1 expression levels change signdficantly during this period. [M=male: F=female]

4.0 DISCUSSION

From the literature it is clear that in the chicken, as in other vertebrates, hyperplastic muscle growth occurs prior to hatching/pre-natally. Muscle growth post-hatch/post-natally is the result of hypertrophy. Previous work from our laboratory suggests that key events in muscle development, and in the sexually dimorphic nature of chicken muscle development, occur between E11.5 and E13.5 of development. The primary objective of the current study is the morphological and molecular characterization of male and female breast muscle at E12.5 of development.

<u>Morphology</u>

We examined the number of myofibres, the cross-sectional area (CSA) of myofibres, and the ratio of PAX7+ve to PAX7-ve cells in sections of breast muscle from five male and five female chicken embryos, at E12.5 of development. Great care was taken to ensure that sections from the same area of breast muscle were analysed in individual embryos. For each sections, five random images were captured. The fifty images captured form five male and five female embryos were then encoded and the different analyses were performed blind. In terms of myofibre morphology, significant differences in myofibre density and myofibre cross-sectional area within defined area were identified between male and female breast muscle on E12.5.

Myofibre density was higher in female chicken breast muscle than male chicken breast muscle. Female breast muscle contained, on average of 18% more fibres per defined area, than male breast muscle. In contrast, measurement of the CSA of individual myofibre revealed that myofibres in male breast muscle are larger than

those in female breast muscle. Male breast muscle myofibres were, on average, 18% larger than female myofibres. A superficial assessment of this analysis would suggest that male breast muscle is composed of a smaller number of bigger myofibres than female breast muscle. However, this analysis was performed on a defined, identically sized area from male and female muscle and when this is considered in light of the fact that a previous study from our group (Sulong, 2016) found that the entire breast muscle in male chick embryos is, on average, 43% larger than that in female chick embryos at E12.5, this suggest that overall, male breast muscle contains a greater number of larger fibres, than the female breast muscle.

We also stained breast muscle sections from five male and five female embryos at E12.5 of development, for PAX7 expression. This analysis revealed that PAX7+ve cells were located both outside and inside the fusing myotubes (Figure 3-8). Secondary myogensis has clearly been initiated and it is likely that these PAX7 positive cells represent fetal myoblasts (Zammit, 2008, Messina and Cossu, 2009) (Figure 1-3). Fetal myoblasts are reported to gradually lose PAX7 expression during the differentiation process (Messina and Cossu, 2009), and therefore, the PAX7 positive cells observed within the myotubes are probably partially differentiated fetal myoblasts which still express PAX7. Our analysis calculated the total number of nuclei and the number of PAX7+ve nuclei within the defined area in sections of male and female breast muscle. This analysis revealed that the ratio of PAX7-positive nuclei to total nuclei is lower in male breast muscle compared to female breast muscle (Figure 3-11). Given that male breast muscle is composed of larger fibre with more nuclei, the lower population of PAX7+ve cells in male muscle, may reflect that a great number of fetal myoblasts have fused with myofibres and differentiated. This

is one possible explanation for the increased size of the myofibres in male breast muscle compared to female chicken breast muscle.

In adult muscle, PAX7 positive satellite cells lie on the surface of myotubes beneath the basal lamina (Mauro, 1961), a location that is consistent with some of the PAX7 positive cells observed here. However, the origin of satellite cells remains controversial. The investigation of myosin heavy chain isoform in different cell culture suggests fetal myoblasts and satellite cells are distinct cell types (Hartley, 1992; Yablonka-Reuveni, 1995). However, it is still unclear whether fetal myoblast can transform into satellite cells during development (Zammit, 2008).

It is not certain exactly when primary and secondary myogenesis occur in the embryonic breast muscle. It has been reported that primary myogenesis in the chicken hindlimb takes place between E5.5 and E8 (Fredette and Landmesse, 1991), however limb musculature develops before the breast muscle and so myogenesis will occur at later stages in breast muscle. The myofibres observed at E11.5 are roughly circular, while those seen at E12.5 have an irregular outline (Figure 3-12). The myofibres present at E12.5 appear to represent fusing myofibres, suggesting that secondary myogenesis initiates at around E12.5. No obvious bundles of myofibres were observed on E11.5, while obvious muscle fascicles has begun to form on E12.5 (Figure 3-12). E12.5 appears to be a unique stage during chicken embryonic myogenesis, both muscle fascicle formation and secondary myogenesis appear to initiate around this stage. The process of secondary myogenesis appear to be complete by E16.5 (Figure 4-1).

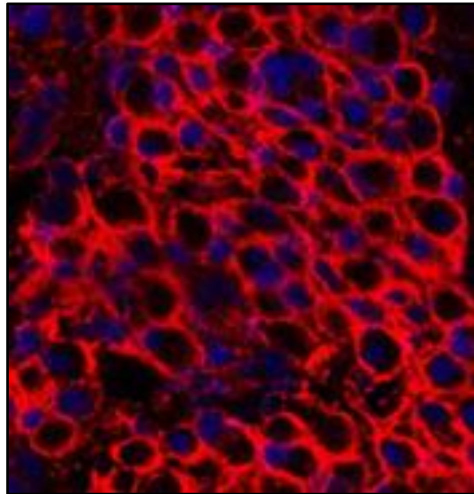

Figure 4-1 Cited from H Sulong, 2016, cross section of chicken breast muscle on E16.5, size bar = 100 µm

Comparing the morphology of tissues in different individual is fraught with technical problems, and even more so, when comparing still-developing embryonic tissues, great care must be taken to ensure that different embryos are at the same stage of development and that the sections compared are from the same area of tissue. In addition, the standard problems of reproducibility of sample and section processing and handling, need to be considered. In order to overcome the majority of these potential problems, we have developed a chimeric muscle chicken embryo model. In principle, chimeric breast muscle will be composed of regions containing a high proportion of male cells and regions containing a high proportion of female cells. This will allow us to compare the morphology of male and female muscle within the same sample, and which, of course, will have been subjected to identical processing, sectioning and handling. This will also enable us to exclude the effect of any hormones and growth factors on sexually dimorphic muscle development, as both male and female muscle regions will have developed within a single individual. Although the examples shown in Figures 3-13 and 3-14 represent preliminary data, it

is clear that this model is realistic and will form the basis of future studies. Complete studies will involve analysing muscle derived from A) female donor and male host, B) male donor and female host, C) female donor and female host, and D) male donor and male host.

Gene expression

In order to gain some insight into the molecular mechanisms underlying sexually dimorphic muscle development, an RNA-seq analysis of gene expression in male and female breast muscle of chick embryos was carried out (Clinton, unpublished). This analysis identified a number of genes that were obviously expressed at higher level in male muscle than female muscle. The majority of these sexually dimorphic genes were associated with either muscle development or neuronal development. Most of the proteins encoded by these genes contribute to structural elements of muscle or neurones. This raised the possibility that male muscle had a higher degree of innervation than female muscle. One of the non-structural sexually dimorphic genes provided a possible mechanism by which male muscle could develop a greater degree of innervation, than female muscle. This gene is MuSK, which encodes the single most important element in NMJ formation (Dechiara et al., 1996; Okada K et al., 2006) and which is expressed at twice the level in male muscle than in female muscle. These findings lead to the hypothesis that male muscle form more NMJ than female muscles, and this leads to a higher proportion of developing myoblasts/myofibres surviving in male embryos, and this in turn results in a greater mass of muscle in male chicken than in female chicken. Although it remains controversial whether secondary myogenesis is dependent on innervation, or not, there is clearly a close link between innervation and muscle survival as denervation

leads to muscle atrophy (Ashby et al., 1993). In addition, motor neurons need neurotrophic factors from muscle cells to survive (DeChiara et al., 1995), and maintaining muscle needs myotrophic factors from neurons (Helgren et al., 1994).

The chromosomal location of the MuSK gene is also relevant to our hypothesis. We have previously shown that the sexually dimorphic phenotype (e.g. muscle mass) is primarily a result of cell autonomous factors, and clearly the molecular basis of this cellular sex identity originates on one or both of the sex chromosomes – Z and W. MuSK is a Z-chromosome gene and is therefore present at twice the copy number in male cells than in female cells. To further explore our hypothesis, we elected to analyse the expression of selected genes in male and female breast muscle at different stages. One of those genes, MuSK, controls NMJ formation but expression is restricted to muscle cells, while the remaining genes were associated with neuronal structure (SNCAIP), neuronal function (DMGDH) and promoting neuronal growth (CNTFR). MuSK expression is significantly higher in male breast muscle than in female breast muscle at all stages of development (Figure 3-15) and the level (per μg RNA) increases throughout secondary myogensis (Figure 3-16). This support the possibility that more NMJ could form in the developing male muscle than in the developing female muscle.

SNCAIP shows a similar sexually dimorphic expression pattern with levels ~2 fold higher in male muscle than in female muscle at all stages of development. SNCAIP encodes synphilin-1, which is a presynaptic protein associated with synaptic vesicles. In young rats, synphilin-1 migrates from the neuronal cell body to the synaptic terminal during development (Ribeiro et al., 2002). The 'per unit' level of SNCAIP (expression per μg RNA) did not alter significantly over the period studied (Figure 3-

18). The higher level of SNCAIP could reflect a greater number of presynaptic termini (and NMJ) in male muscle than in female muscle.

DMGDH expression showed a similar relative expression profile to that seen with MuSK and SNCAIP, with levels approximately 2 fold higher in male breast muscle than in female breast muscle at all stages studied. This sexually dimorphic expression would be consistent with a greater number of neurons present in male muscle than in female muscle. The 'per unit' level of DMGDH increased substantially throughout secondary myogenesis and could reflect an increase in neuronal metabolism/activity.

In contrast to MuSK, SNCAIP and DMGDH, the expression level of CNTFR did not display a consistent sexually dimorphic pattern. During most of the secondary myogenesis period, CNTFR expression was similar in male and female breast muscle, and the expression per µg RNA did not change over this period. Only at E12.5 did CNTFR display sexually dimorphic expression, with higher levels in male breast muscle than in female breast muscle. This could further support the proposal that E12.5 is a key period in muscle development and could reflect increased neuronal growth at this stage in male muscle. CNTFR is critical in muscle-derived neurotrophic activity, and this activity ensures that only neurons forming correct NMJ with muscle fibre can survive (DeChiara et al., 1995).

BMP signalling

The bulk of studies relating to MuSK, demonstrate that this protein is the crucial element in assembling the post-synaptic complex required for NMJ formation. However, it has recently been reported that, in certain circumstance, MuSK can act as a co-receptor of BMP signalling (Yilmaz et al., 2016). Given that BMP signalling

is a significant signalling pathway in muscle (Sartori et al., 2013), this raises the possibility that the sexually dimorphic expression of MuSK that we have demonstrated in chick embryo breast muscle, relates to BMP signalling rather than, as we propose, NMJ formation. To explore this possibility, we examined BMP signalling in male and female chick embryo breast muscle at three different levels; 1) at the level of ligand and receptor, 2) the phosphorylation of signal transduction factors, and 3) the expression of MuSA1, an E3 ubiquitin ligase associated with muscle atrophy and known to be regulated by BMP signalling.

First, we measured expression of BMP2, BMP4, BMP7 and BMPR1B in breast muscle of five embryos at the key E12.5 stage of development. With the exception of BMP2, expression levels were similar in male and female muscle. BMP2 was expressed at significantly higher in male breast muscle than in female breast muscle. BMP2 has been shown to stimulate bone production and induce osteoblast differentiation (Marie et al., 2002; Urist, 1965). Further investigation is required to elucidate the role BMP2 plays in skeletal muscle development.

Future work in this area will involve characterising the expression of BMP2 in male and female muscle throughout secondary myogenesis, and obtaining a BMP2 antibody to localise the signal by immunostaining.

Following receptor binding, BMP signalling is transduced via the phosphorylation of the RSMADS (typically, SMAD1, 5 and 8). To assess the level of BMP signalling in male and female breast muscle, we used an antibody against the phosphorylated SMAD 1/5/8 complex. We used this antibody to immunostain male and female breast muscle sections from chick embryos at E12.5 and E16.5 (Figure 3-24, Figure

3-25). This analysis suggest that the most intense pSMAD signal was confined to the connective tissue regions of male and female muscles. This assessment was confirmed by co-staining for PAX7, where the signal is restricted to the myofibre region. This suggests that participation in the formation and maintenance of NMJs may still be the primary function of MuSK, and BMP signalling may influence muscle development via cells in the connective tissue, rather than by regulating myofibres directly.

Finally, we measured the expression of MuSA1 in breast muscle from five male and five female embryos at different stages of development. MuSA1 levels were highest at E8.5 and considerably reduced during the key stages of secondary myogenesis (Figure 3-27), E10.5-E14.5 (as much as 20-fold). This suggests that in both male and female breast muscle, BMP signalling is increased over the period E10.5-E14.5 (BMP signalling negatively regulates MuSA1 expression). There was also evidence of sexually dimorphic expression (Figure 3-26): MuSA1 was expressed at higher levels in male muscle at E8.5 (primary myogenesis) and at higher levels in female muscle at E12.5 and E14.5 (secondary myogenesis). Overall, this analysis suggests that BMP signalling increases significantly during the period of secondary myogenesis, and that BMP signalling influences muscle development via factors originating within the connective tissue element of the breast muscle.

In addition to reflecting changes in BMP activity, the variation in MuSA1 expression levels will also reflect changes in protein ubiquitination and turnover. The higher MuSA1 expression levels seen at E8.5 is possibly due to the degradation of primary myotubes that occurs during primary myogenesis. It has been reported that a proportion of primary myotubes degrade within 3 days of their formation (Ashby et

al., 1993). The higher level of MuSA1 seen in female muscle compared to male muscle at E12.5 and E14.5 may reflect an increased atrophy in female muscle, and would be consistent with our hypothesis that in male muscle a greater proportion of myofibres survive as a result of a higher degree of innervation.

The higher levels of MuSA1 seen in male and female breast muscle at E16.5, may reflect a decrease in BMP signalling compared to E10.5-E14.5, or it may reflect the increase in muscle cytoplasmic protein turnover that occurs over the period E16.5 to hatch (E21).

Over the period E14.5-E16.5, the mass of growing breast muscle equalises in male and female embryos and this is thought to be due to space restriction imposed by the rigid eggshell. Between E16 and hatch, the breast muscle mass of both male and female chick embryos is reported to decrease (Chen et al., 2013). This is thought to be due to the requirement for a novel source of nutrients following exhaustion of the yolk as the primary source of nutrients. For the final period of embryonic development, breast muscle cytoplasm is utilized as a source of nutrients. This is specific to breast muscle and does not affect other muscle – this is thought to be due to the fact that breast muscle is not absolutely required in the immediate post-hatch period. The breast muscle recovers rapidly post-hatch when alternative nutrients are available. Post-hatch muscle growth is hypertrophic and male develop a greater mass of muscle than female due to an increased number of myofibres. Our hypothesis is that male contains a higher number of myofibres because a greater degree of innervation allows more myofibres to survive during secondary myogenesis.

5.0 CONCLUDING REMARKS AND FUTURE WORK

Adult male chickens have a greater mass of breast muscle than adult female chickens, and the poultry industry has established that male birds have a greater food conversion ratio (FCR) – at six weeks of age, male birds are 35% heavier than female birds, on an identical feed intake. The difference in muscle mass must be due to A) males possessing a greater number of myofibres, B) males having the same number of larger myofibres, or C) a combination of both A and B. Our morphological analyses suggest that at a key stage of secondary myogenesis (E12.5), male breast muscle contains a greater number of myotubes undergoing fusion and differentiation than female muscle. If this situation persists post-hatch (more fibres with more nuclei), then male breast muscle would have a greater potential for hypertrophic growth than female breast muscle. Future work in this area will include repeating our morphology study on an array of chimeric muscle samples, and analysis of the CSA, myofibre number all nuclei number in male and female breast muscle from post-hatch chicken and adult birds.

An RNA-seq study identified the sexually dimorphic expression of in a number of genes associated with innervation. One of these, MuSK is the key factor required for neuromuscular junction (NMJ) formation. As innervation and muscle maintenance are intimately linked, it is possible that the greater mass of muscle seen in male birds is due to more myofibres surviving in male embryos as a result of more NMJ forming. Initially we hoped to quantify the number of NMJs in male and female muscle, but this proved impractical. However, our gene expression analyses are consistent with our hypothesis: MuSK is expressed at higher level in male muscle

than in female muscle at all stages of development and the expression profile of SNCAIP and DMGDH are consistent with a greater number of active neurones in male muscle than in female muscle. The higher levels of CNTFR found in male muscle compared to female muscle at E12.5 is also consistent with the generation of a greater number of neurones in male muscle at this stage of development. Future work in this area will include developing protocols to enable quantitation of NMJ in muscle from late embryonic stages and post-hatch muscle – bungarotoxin staining on longitudinal sections. We will also use our established CRISPR/transgenic technologies to generate a line of male birds containing only a single copy of the Z-chromosome MuSK gene. In theory, the reduced levels of MuSK expression should result in a male bird of female proportions. We will also generate an antibody to chicken MuSK for protein analysis throughout development.

Our analyses also investigated the possibility that the sexually dimorphic expression of MuSK is related to BMP signalling as supposed to NMJ formation. Although our analysis suggested that BMP signalling is important in muscle development and is increased dramatically during secondary myogenesis, it suggests that MuSK and BMP signalling are not directly related. Most BMP activity was associated with the connective tissue region of muscle, whereas MuSK is well established as restricted to myotubes/myofibres. As part of this study, we measured expression of the E3-ubiquitin ligase, MuSA1. The higher levels of MuSA1 expression seen in female muscle compared to male muscle at E12.5 and E14.5 are consistent with higher level of atrophy (reduced survival) in female muscle than male muscle during secondary myogenesis.

6.0 ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Michael Clinton and Dr. Tom Burdon for their guidance during the whole year of my project as well as helping me improve my language. Mike always listened to my opinion patiently and then gave me reasonable guidance and suggestions when I met problems during my research. With his help, I gradually understood what science is and what scientific research should be.

I also want to express my thanks to Dr. Debiao Zhao for teaching me all the techniques involved in my study, with great patience and enthusiasm. The chimeric muscle was also kindly provided by him. Without Debiao, I could not have finished my project. With his help and advice I became more careful and patient with my experiment.

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Thanks all my friends in the Roslin Institute. I really had a great time.

7.0 REFERENCE

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