Satellite Cell and Myonuclear Distribution within Normal and Hypertrophic Models of Skeletal Muscle Growth, and the Expression of Myogenic Regulatory Factors during Growth

A thesis submitted to the College of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Anatomy and Cell Biology, University of Saskatchewan Saskatoon

By

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of the requirements for the

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by

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ABSTRACT

Satellite cells (SCs) are mononuclear myogenic stem cells located between the basal lamina and plasmalemma of the skeletal muscle fiber. They are responsible for postnatal skeletal muscle growth, repair and regeneration. Once activated, SCs divide and fuse to the muscle fibers where their nuclei become new myonuclei. Earlier studies suggested that SCs were either randomly or evenly distributed along muscle fibers. However, myonuclei were found to be more concentrated at tapered ends of muscle fibers. Myogenic regulatory factors, mainly MyoD and Myogenin, are expressed by active SCs. Previous *in vitro* and prenatal studies suggested that MyoD expression demarcates the end of proliferation while Myogenin can demarcate the differentiation stage within myoblasts. Few *in vivo* studies have reported the expression of MyoD and Myogenin within SCs, and none have attempted to discern their expression patterns during growth. Meat producing chickens represent a unique model for natural hypertrophy within muscle fibers. However, very little is known about the distribution of SCs within these naturally hypertrophied fibers, and whether this distribution is comparable to that of experimental models of hypertrophy. Nandrolone Decanoate is the anabolic steroid most commonly used to increase skeletal muscle mass and strength, although little is known of its effects on SCs. This thesis expands our understanding of SCs by examining the following hypotheses: 1) there is a greater frequency (number of SC nuclei over all nuclei within the basal laminae) and a higher concentration (less surface area of sarcolemma per SC) of SCs at the ends of developing skeletal muscle fibers, 2) MyoD and Myogenin transcription myogenic factors have distinctive patterns of expression within SC

nuclei during maturation, 3) there are greater frequency and concentration of SCs and greater number of myonuclei in naturally hypertrophied muscle fibers compared to their control, and 4) there is a greater frequency and a greater concentration of SCs in Nandrolone treated birds than in controls. Chicken pectoralis muscle was the main experimental model used in this thesis because of its overlapping fibers arranged in series, the presence of neonatal myosin at the fiber ends and relative homogeneity of fiber type. Immunocytochemical techniques that include an antibody against Pax7 to identify SC nuclei were applied, and computer image analyses were then used to quantify the numbers of SC nuclei and myonuclei within muscle fibers. This thesis demonstrates that throughout development there is a greater frequency and concentration of SCs at the ends of developing skeletal muscle fibers, which indicates a major contribution of these cells in the longitudinal growth of muscle fibers. It also reveals that MyoD and Myogenin each has a distinctive pattern of expression within SCs during *in vivo* postnatal development. The expression of MyoD increases significantly during maturation, while Myogenin expression remains steady. This finding suggests that each of these myogenic factors play a different role in the postnatal activation of SCs. Lastly, it is the first study to show a greater frequency and a higher concentration of SCs within both naturally and Nandrolone induced hypertrophied muscle fibers. This indicates SCs may be critically involved during postnatal skeletal muscle growth and hypertrophy.

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LIST OF ABBREVIATIONS

AAS	anabolic androgenic steroids
ALD	anterior latissimus dorsi muscle
C-met	cell surface tyrosine kinase receptor for HGF
DAPI	4',6 diamidino-2-phenylindole
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycoltetraacetic acid
HGF	hypatocyte growth factor
IGF-1	insulin like growth factor 1
MyHC	myosin heavy chain
MN	myonuclei
NO	nitric oxide
NP-40	nonidet P-40
Pax7	paired box transcription factor seven
PB	phosphate buffer
PBS	phosphate buffer saline
PMSF	phenylmethylsulphonyl fluoride
RCF_{max}	relative centrifugal force maximum
SC	satellite cell
SCN	satellite cell nuclei

SD	standard deviation
SDS	sodium dodecyl sulfate
SE	standard error

1.0 CHAPTER

GENERAL INTRODUCTION

1.1 Satellite cells

Muscular tissue is considered one of the four basic tissues in the body. It is further divided into three types of muscles; smooth, cardiac and skeletal. Smooth muscles are found around hollow tubes in the body, such as the intestines, and have a high regeneration capability through either division or differentiation. Cardiac muscle forms most of the heart and is considered to have no capacity for regeneration. Skeletal muscles are the main part of the muscular tissue and are responsible for movement and support. The regeneration capability of skeletal muscles has been described as limited, and due to a specific population of progenitor adult stem cells known as satellite cells (SCs; Gray and Carter 2005; Gartner and Hiatt 2007).

SCs are mononucleated myogenic stem cells located between the basal lamina and plasmalemma of the skeletal muscle fiber, and are responsible for postnatal muscle growth and regeneration (Muir 1970; Morgan and Partridge 2003; Mckinnell et al. 2005). SCs were discovered in 1961 by Mauro and Katz while studying the skeletal muscles of the frog (Mauro 1961; Katz 1961). The SC is fusiform-shaped with a central nucleus, with its long axis directed along the length of the muscle fiber (Fig. 1). A SC can reach 25 μm in length but is less than 5 μm in width, and possesses elongated cytoplasmic tails at each end (Muir 1970; Castillo de Maruenda and Franzini-Armstrong 1978; Novotova and Uhrik 1991).

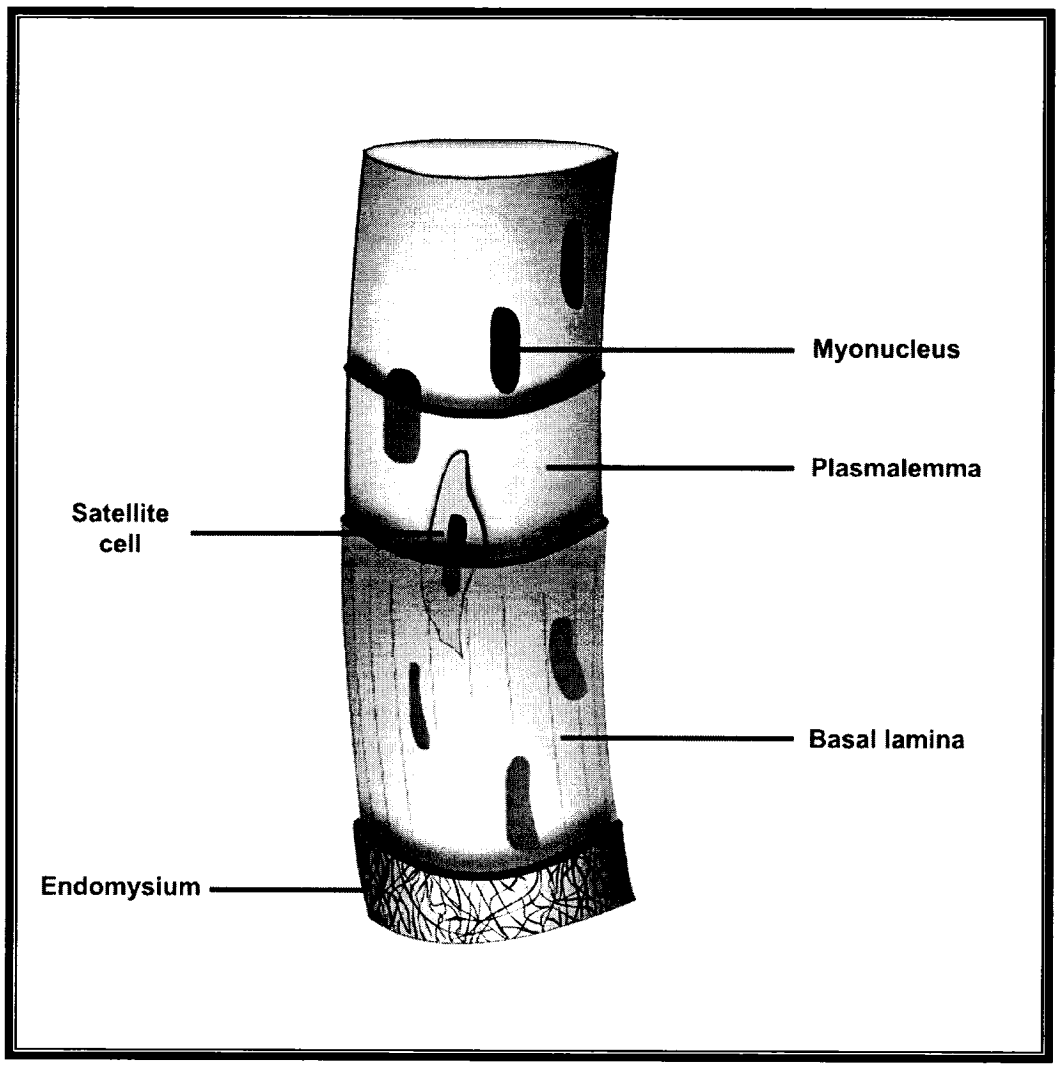

Figure 1: A satellite cell (SC) and myonuclei within a muscle fiber. This diagram shows from deep to superficial, the plasmalemma, basal lamina and endomysium layers that cover the muscle fiber. Myonuclei are located within the fiber beneath the plasmalemma, while the SC is sandwiched between the plasmalemma and basal lamina.

During myogenesis, myogenic precursors differentiate from mesodermal cells of the somite and migrate to their final destination. After that, myoblasts start fusing together to form myotubes which become muscle fibers. Some of these myogenic precursors do not differentiate into myotubes, but instead reside beneath the muscle fiber basal lamina to become SCs after birth (Hawke and Garry 2001; Mckinnell et al. 2005; Zammit et al. 2006a). It is unclear whether SCs originate from the somite as a distinct lineage of myogenic precursors, or as a preexisting lineage such as embryonic or fetal myoblasts. It has also been suggested that SCs can develop from a non-somitic origin such as endothelial cells or a precursor common to both SCs and endothelial cells (De Angelis et al. 1999, Seale and Rudniki 2000).

SCs can be recognized based on their distinctive position between the basal lamina and plasmalemma of the muscle fiber. An immunostaining technique can be used to outline the plasmalemma (by labeling the dystrophin protein; McLoon et al. 2004), basal lamina (by labeling the glycoprotein laminin; Halevy et al. 2004), and all nuclei (by using a DNA stain such as DAPI or Hoechst). Any nucleus between the plasmalemma and basal lamina is defined as a SC nucleus. In addition to this location, many protein and molecular markers have been used to identify SCs. The paired box transcription factor seven (Pax7) has been one of the most common markers used for this purpose. Pax7 is a highly conserved protein that belongs to the helix-turn-helix class of transcription factors. It was found to be expressed specifically in cells residing beneath the basal lamina in positions characteristic for SCs (Seale et al. 2000; Collins et al. 2005; Relaix et al. 2005; Allouh and Rosser

2006). SCs were also absent in Pax7 null mice, which suggests a crucial role for Pax7 in specification of SCs (Seale et al. 2000). Pax7 is found to be up-regulated in SCs that exit the cell cycle and yet escape differentiation (Olguin and Olwin 2004). Other markers used to label SCs include M-Cadherin, myocyte nuclear factor and CD34 (Morgan and Partridge 2003). The advantages of Pax7 over other markers are that it is expressed in all SC nuclei and effective antibodies are readily available (Zammit et al. 2006a).

During postnatal growth, SCs are usually active. They undergo proliferation and differentiation by fusing to growing muscle fibers where their nuclei become new myonuclei (Morgan and Partridge 2003; Halevy et al. 2004). In adult life, however, SCs are quiescent. In response to injury of muscle fibers, SCs may become active and start expressing myogenic regulatory factors. They migrate to the site of injury and enter the proliferation and differentiation cycle again where they fuse to the injured muscle fibers or, to a lesser extent, fuse together forming new fibers. Nevertheless, some of these proliferating SCs do not differentiate but return to quiescence as a self renewal mechanism (Fig. 2; Hawke and Gary 2001; Zammit et al. 2006a).

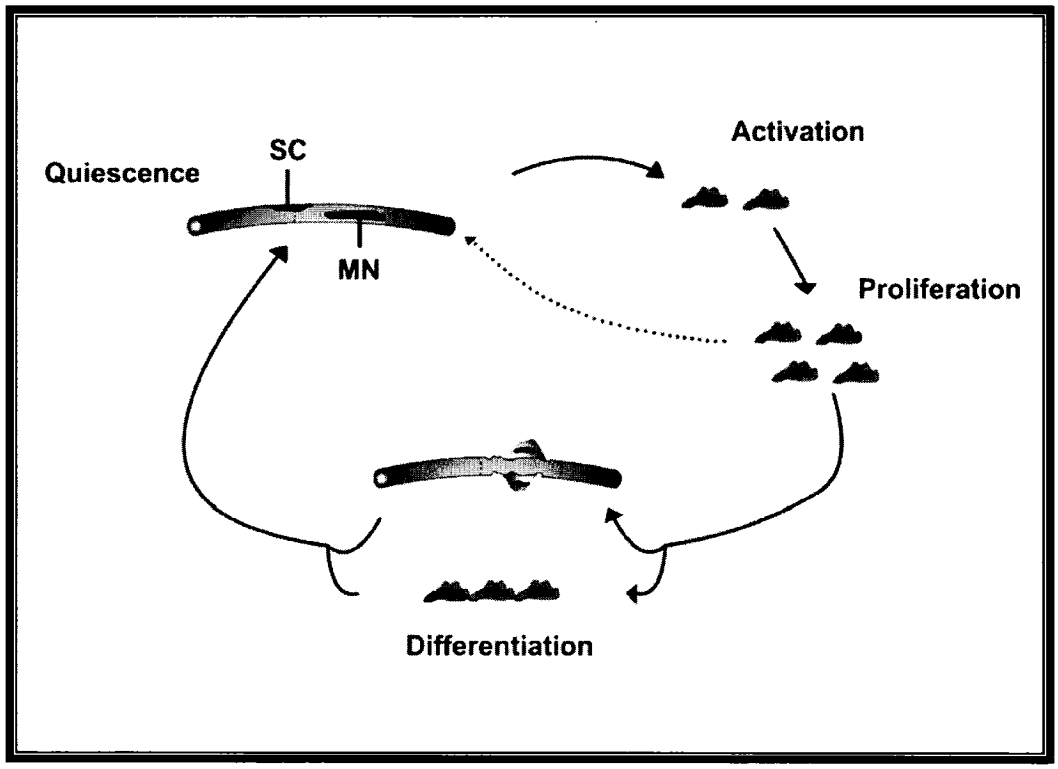

Figure 2: The life cycle of satellite cells (SCs). SCs are usually quiescent and located beneath the fiber's basal lamina. Under an appropriate stimulus, they become active and start to express the different myogenic factors. Their cytoplasmic volume increases significantly with well developed organelles. After that, SCs start to proliferate, and then differentiate by either fusing to a preexisting fiber or by fusing together to form a new fiber. Eventually, SC nuclei become new myonuclei. However, a small proportion of proliferated SCs will not differentiate. Instead they return to quiescence to reestablish the pool of SCs.

The molecular mechanism for activation of SCs starts by the synthesis of nitric oxide (NO) from L-arginine within injured muscle fibers (Reid 1998; Anderson 2000; Wozniak et al. 2005). NO reacts on its cytoplasmic receptor guanylyl cyclase to produce cyclic guanylyl monophosphate (Ignarro et al. 1980; Wedel and Garbers 2001). This leads to the release of hepatocyte growth factor (HGF). HGF then binds to its c-met receptors on the surface of SCs, which initiates the mitogen-activated protein kinase pathway and the phosphatidyl inositol 3 kinase pathway (Cornelison and Wold 1997; Furge et al. 2000; Wozniak et al. 2005). These two pathways induce the transcription and translation of myogenic regulatory factors and other transcriptional factors required for activation and proliferation of SCs (Kami et al. 1995; Wozniak et al. 2005).

The distribution and frequency of SCs (number of SC nuclei over myonuclei and SC nuclei) in skeletal muscles has been correlated with several factors such as species, age, type of muscle fiber and presence of specific anatomic structures like motor end plates and blood capillaries. For example, the frequency in sartorius muscle of the pig was found to be 0.01 (Hawke and Gary 2001), while in frog sartorius it was 0.10 (Castillo de Maruenda and Franzini-Armstrong 1978). While SCs constitute about 30% of muscle nuclei in newborn rodents, this ratio is decreased to 4% in adults and 2% in senile animals (Snow 1977).

The frequency of SCs is different among fiber types, as within each species studied there are more SCs in red (oxidative) fibers than in white (glycolytic) fibers

(Gibson and Schultz 1983). The myonuclear density of the red fibers is greater than that of white fibers, which means red fibers possess more nuclei than white fibers of the same size (Schultz and McCormick 1994; Tseng et al. 1994; Schmalbruch and Lewis 2000). The exact reason why red fibers require more nuclei is not known, but it could be related to a higher metabolic activity and higher protein remodeling rate in red than white fibers. The SC population in a muscle always reflects the requirement for myonuclei production (Schultz and McCormick 1994). Since the mitotic rate (activity) of SCs in both types of fibers is similar, the only compensatory measure to add more myonuclei in red fibers is to have additional SCs (Schultz and McCormick 1994).

SCs are more concentrated near specific anatomic structures such as motor end plates and blood capillaries (Schultz and McCormick 1994). Previous studies state that SCs are either evenly (Snow 1981) or randomly (Novotova and Uhrik 1991) distributed along the fiber length with greater numbers near motor endplates. However, some of these studies (Snow 1981) mention that the number of SCs around myoneural junctions is not large enough to significantly affect the mean percentages of SCs. Other work indicates that SCs are more numerous near blood capillaries (Schmalbruch and Hellhammer 1977), as are myonuclei (Ralston et al. 2006). Nonetheless, many questions remain concerning the distribution of SCs in muscle fibers.

1.2 The myonuclear domain

Skeletal muscle fibers are comparatively large cells where a single fiber can have a total volume of several microliters. The muscle fiber maintains its large volume of sarcoplasm by having hundreds to thousands of nuclei. An earlier study had suggested that products of myonuclei can mix in the muscle fiber (Frair and Peterson 1983). However, later experiments have suggested that most of the proteins are locally synthesized, processed and distributed within fibers (Pavlath et al. 1989; Ralston and Hall 1989; Rotundo 1990). This later suggestion supports the theory of myonuclear domain, where each myonucleus is responsible for gene expression within its surrounding portion of sarcoplasm.

The size of the myonuclear domain is the volume of sarcoplasm that is associated with a single myonucleus. It has been suggested that myonuclear domain size is correlated with the skeletal muscle fiber type and the rate of protein synthesis during development (Edgerton and Roy 1991). Previous studies have found a smaller domain size in slow type I fibers than fast type II fibers (Edgerton and Roy 1991; Tseng et al. 1994; Kasper and Xun 1996b). This is due to the smaller fiber size and higher myonuclear number in slow type I fibers.

Regarding the rate of protein synthesis, there is disagreement in the literature on whether the domain size changes during muscle development. Some studies have claimed that during postnatal development the number of myonuclei increases to maintain a constant myonuclear domain size (Moss 1968; Winchester and Gonyea

1992; Allen et al. 1995). Other studies indicate that despite the increase in myonuclear number, the domain size also increases during normal development (Knizetova et al. 1972; Mozdziak et al. 1994, 1997).

1.3 Expression of myosin heavy chain isoforms

The myosin protein, which forms the thick myofilaments in skeletal muscle fibers, is composed of two heavy chains and four light chains (Lowey 1994; Tidyman et al. 1997). It makes up more than 50% of skeletal muscle protein (Craig and Padron 2005). Myosin thick filaments constitute a major component of the muscle contractile unit. It is well established that muscle contraction occurs through binding of the heads of myosin heavy chains with thin actin filaments (Craig and Padron 2005).

Skeletal muscle fibers are classified based on their myosin heavy chain (MyHC) isoform complement (Schiaffino and Reggiani 1996; Spangenburg and Booth 2003). Slow type I fibers contain type I myosin, while type II fast-twitch fibers contain type II MyHC isoform. In mature mammalian muscle, type II fibers are further subclassified into type IIa, type IIb and type IIx fibers. Each of these fibers contains a specific MyHC isoform that determines the velocity of contraction and ATPase activity within the fiber (Weiss et al. 1999; Talmadge 2000; Pette and Staron 2001). The speed of muscle fiber contraction according to MyHC isoform is described as IIb > IIx > IIa > I. In mammals, during development, there is also an

embryonic and a neonatal isoform that are subsequently replaced by these mature adult isoforms.

Avian skeletal muscles contain both fast and slow MyHC isoforms. The fast isoforms include three embryonic, a neonatal and an adult isoform (Tidyman et al. 1997). During development, only few muscles replace the embryonic and neonatal isoforms by the adult isoform. Other avian muscles never express the adult isoform, instead they retain either the embryonic or the neonatal isoform (Bandman and Rosser 2000).

Distinct MyHC isoforms are sequentially expressed in chicken pectoralis muscle during various stages of development. Embryonic isoforms are directly replaced by the neonatal isoform that is in turn gradually replaced by an adult isoform (Bandman et al. 1994; Rosser et al. 1995; Bandman and Rosser 2000). These isoforms are functionally related, but differ in their biochemical structure due to some variation in amino acid sequence. The myosin transformation process in the fibers of chicken pectoralis is initiated near the centrally located motor end plate and progresses towards the fiber tapered ends (Rosser et al. 2000). As the adult isoform starts to be expressed during the early post-hatch period, some regions between adult and neonatal myosin overlap. These regions can be defined as transforming (Rosser et al. 2000). In later post-hatch development, the neonatal isoform is progressively restricted to and retained within the tapered ends of chicken pectoralis fibers (Fig. 3).

As the transformation begins centrally in the thicker regions of the fibers, and progresses to the more peripheral tapered ends, the adult fiber profiles seen in a cross-section of the muscle represent the central regions of the fiber, and the neonatal profiles are the fiber ends. The transforming profiles are from regions between these adult and neonatal profiles. The fiber profiles show the following gradation in size: adult > transforming > neonatal (Rosser et al. 2000, 2002).

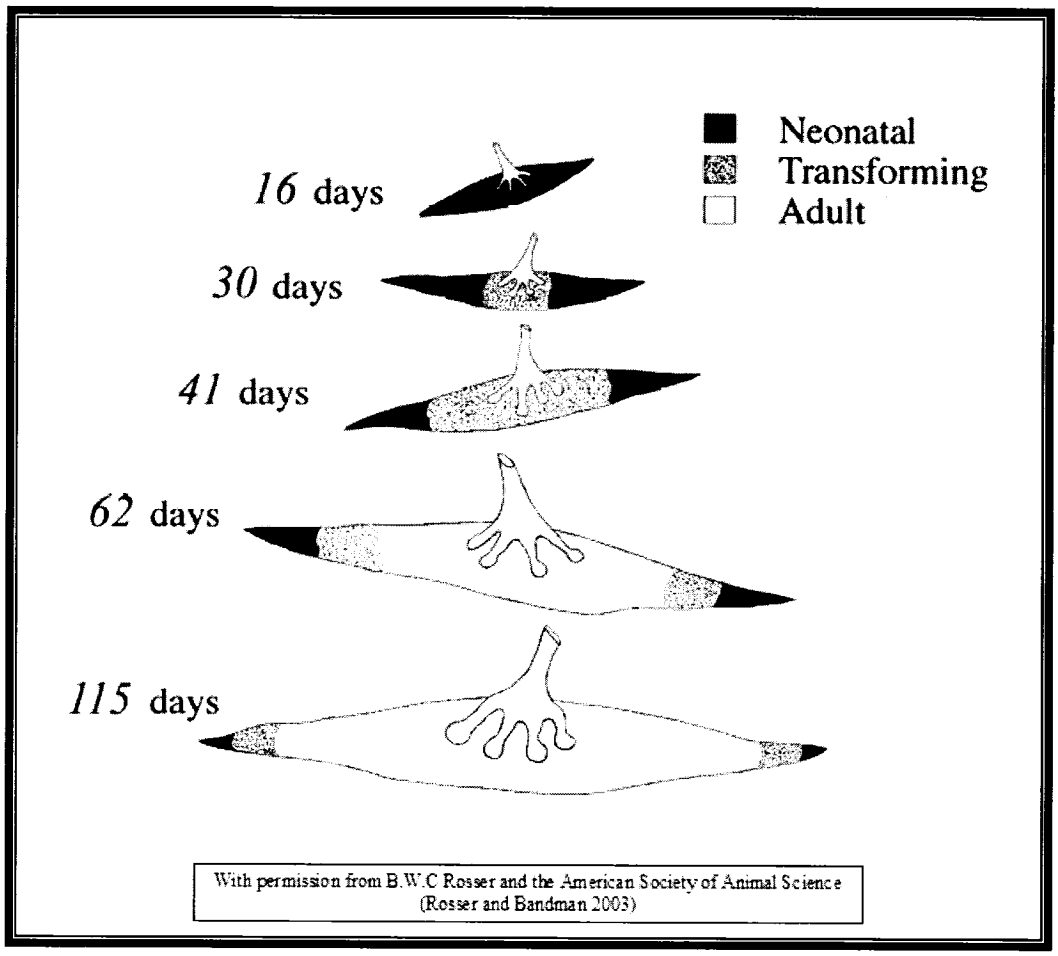

Figure 3: Transformation of myosin heavy chain isoforms within chicken pectoralis. At age 30 days post-hatch, the adult myosin isoform starts to replace the neonatal isoform near the centrally located motor end plate. The transformation process progresses with age toward fiber ends until the neonatal isoform is restricted to the fiber tapered ends near maturation. The regions that contain both isoforms where the transformation process is still underway are defined as transforming regions.

1.4 Anabolic androgenic steroids

Anabolic Androgenic Steroids (AAS) are synthetic drugs that mimic the primary male sex hormone testosterone. Testosterone is responsible for both the androgenic and anabolic effects that take place during puberty and continue in adulthood. The androgenic effects of testosterone during puberty include the activation of the male reproductive system and the development of secondary male sexual characteristics such as hair distribution and deepening of the voice. The anabolic effects include an increase in height and an increase in muscle mass and strength. AAS are the consequence of research to develop drugs that would separate the anabolic capability of testosterone from its androgenic properties. However, this separation has never been fully achieved and no purely anabolic agent has ever been synthesized (Kochakian 1976; Gunes and Fetil 2000; Clark and Henderson 2003).

There are over 100 AAS available (Sullivan et al. 1999). They all share the same basic chemical structure of a four-ring carbon skeleton. The main differences in chemical structure are the number of carbon atoms attached to number 17 carbon atom in the skeleton and the manner of how hydrogen, oxygen and hydroxyl groups are attached to carbon atoms. These chemical differences have been induced to enhance the anabolic effects and reduce the androgenic properties of these drugs (Wright and Cowart 1990).

AAS are lipid soluble molecules that can pass easily through membranes to bind to their androgenic receptors within the nucleus. Nuclear androgenic receptors

are transcription factors with a ligand binding domain and a DNA binding domain. Binding of AAS to their receptors regulates the transcriptional activity of these receptors, which in turn regulates the expression of several target genes (Cooper and Hausman 2004). Within skeletal muscle tissue, androgenic receptors have been found in both myonuclei and SC nuclei (Sinha-Hikim et al. 2004). This finding supports the suggestion that AAS are acting through more than one mechanism to enhance the growth of skeletal muscle fibers.

AAS have been classified into three categories based upon their interaction with nuclear androgenic receptors (Saartok et al. 1984). The first includes AAS that bind with high affinity to androgen receptors. These steroids are described as strong androgens, and include most of the synthesized forms of testosterone. The second category represents steroids that bind with low affinity to androgen receptors. These steroids, like stanozolol, are described as weak androgenic substances. The third category contains those AAS that do not bind to androgen receptors at all. These steroids act after biotransformation to other compounds. Oxymetholone is an example of this group of steroids (Toth and Zakar 1982; Saartok et al. 1984).

The exact molecular mechanism of action of AAS is not completely understood and still subject to research. Several mechanisms and theoretical models of action have been suggested. For example, the enzyme 5-α-reductase has been recognized as playing an important role in the action of AAS (Wilson 1988). This enzyme converts most AAS into the more active compound dihydrotestosterone

within target cells. 5-α-reductase activity is located primarily in male sex glands, skin and fat cells. However, skeletal muscle possesses low 5-α-reductase activity (Wilson 1988). Another suggested molecular mechanism of AAS action within skeletal muscle is the enhanced transcription of insulin-like growth factor 1 (IGF-1) by the AAS-receptor complex (Lang and Frost 2002). IGF-1 then acts via two mechanisms to enhance skeletal muscle growth and hypertrophy. First, IGF-1 increases protein synthesis within muscle by activating translational initiation (Bodine et al. 2001). Second, IGF-1 inhibits p27 cyclin-dependent kinase inhibitor protein thereby signaling SCs to proliferate (Chakravarthy et al. 2000). The p27 protein inhibits cyclin-dependent kinase 2, which is a necessary enzyme for transmitting SCs toward the proliferation stage.

AAS abuse exerts many side effects including both physiological and behavioral problems. These side effects are mainly related to the dosage, duration and type of steroid being taken (Wu 1997). The physiological problems include cardiovascular effects, such as cardiomyopathy and impaired diastolic function (Kennedy and Lawrence 1993; Sullivan et al. 1999). Also, raised blood pressure is one of the most common side effects of AAS misuse (Lenders et al. 1988; Perry et al. 1990). Another physiological problem related mainly to chronic abuse of oral steroids is liver toxicity. Nearly all studies of AAS users have documented abnormalities in liver function (Hickson et al. 1989; Friedl 1993; Yesalis and Cowart 1998). Boada et al. (1999) studied the hepatic effects of high doses of the anabolic

steroid stanozolol in rats. They found that the liver exhibited inflammatory and degenerative lesions in centrilobular hepatocytes.

The most noticed behavioral side effect of AAS abuse is aggression leading to violent outbursts (Pope and Katz 1990; Corrigan 1996). Steroid abusers usually become very aware of their surroundings, and this heightened awareness can sometimes lead to schizophrenic and psychotic episodes (Annitto and Layman 1980; Choi and Pope 1994; Stanley and Ward 1994). Also, AAS users generally report an increased sexual drive (Lenehan et al. 1996). It is believed that the androgenic effects of the steroids enhance this sexual desire (Moss et al. 1993).

The most popularly used injectable steroid is Nandrolone Decanoate, which is also known as Deca Durabolin. Nandrolone causes muscle to store more nitrogen than it releases, so a positive nitrogen balance is achieved. The positive nitrogen balance is synonymous with muscle growth because it allows the muscle fiber to accumulate a larger amount of protein than usual (Van Wayjen 1993). Since its initial discovery in 1970s, Nandrolone has been used in the treatment of several clinical conditions, and is also used by athletes to enhance their competitive capabilities (Wright 1982; Lenehan 2003; George 2005).

2.0 CHAPTER

SPECIFIC AIMS

SCs play a critical role during post-natal growth, repair and regeneration of skeletal muscles. Their function and distribution depend on several factors such as fiber type, age and the situation in which they are active or quiescent. However, despite the vast body of ongoing research in this field, investigations are still required in a number of areas regarding SCs. This thesis examined four main aspects of SCs in developing skeletal muscle fibers.

The specific aims are:

1) To delineate the distribution of SCs both temporally and spatially during development. It is hypothesized that the frequency of SCs is decreased with age, and there is a greater frequency and a greater concentration of SCs at the ends of muscle fibers. Anti-Pax7 antibody was used to identify SC nuclei in their characteristic positions beneath muscle fibers basal laminae in the chicken pectoralis muscle. The frequency of SCs with age, and the frequency and concentration of SCs along the fibers length were then measured.

2) To clearly characterize the *in vivo* expression patterns of both MyoD and Myogenin myogenic regulatory factors by SC nuclei during growth. It is hypothesized that MyoD and Myogenin transcription factors have distinctive patterns of expression within SC nuclei during maturation. The percentages of SCs that express MyoD and/or Myogenin within chicken pectoralis muscle were obtained from several ages during post-hatch development until adulthood. The percentages of

myonuclei that express MyoD and/or Myogenin were also obtained from the same ages.

3) To determine the frequency and concentration of SCs within hypertrophied muscles of meat producing chickens that represent a natural model of hypertrophy. It is hypothesized that there are a greater frequency and concentration of SCs and greater number of myonuclei in hypertrophied muscle of a natural model. A secondary aim is to compare the distribution of SCs in this natural model with other experimental models of hypertrophy studied previously.

4) To better understand the effects of the anabolic steroid Nandrolone Decanoate on the distribution of SCs, and to determine its effects on the morphology of skeletal muscle fibers such as fiber size, the distribution of myonuclei and size of myonuclear domains. The thesis tests the hypothesis that Nandrolone administration leads to increased muscle mass and fiber size within the growing chicken pectoralis, and the hypothesis that there is a greater frequency and a greater concentration of SCs in Nandrolone treated birds than in the controls. The frequency and concentration of SCs were measured in the pectoralis muscle of two groups of chickens. The first group received Nandrolone injections and the second received normal saline as a control. In addition to SC distribution, muscle weight, fiber size and size of myonuclear domains were measured and compared between Nandrolone treated and control groups.

3.0 CHAPTER

GREATER FREQUENCY AND CONCENTRATION OF SATELLITE CELLS AT THE ENDS OF GROWING SKELETAL MUSCLE FIBERS

3.1 Introduction

Satellite cells (SCs) are mononuclear myogenic cells located between the basal lamina and plasmalemma of the skeletal muscle fiber (Mauro 1961; Schultz and McCormick 1994; Wozniak et al. 2005). They contribute to skeletal muscle growth, regeneration and repair (Lemischka 1999; Charge and Rudnicki 2004). Under proper stimuli, such as injury to the muscle fiber or exercise, SCs can become active, proliferate and fuse with the fiber to become new myonuclei (Moss and Leblond 1971; Seale and Rudnicki 2000; Hawke and Garry 2001). The paired box transcription factor seven (Pax7) is expressed by quiescent, active and proliferating SCs, but is not expressed by myonuclei (Seale et al. 2000; Collins et al. 2005; Relaix et al. 2005). Pax7 is an upstream transcriptional activator of the myogenic regulatory factor MyoD, and also plays a critical anti-apoptotic role in activated SCs (Reliax et al. 2006). The specific expression of Pax7 by all SCs in the pectoralis muscle of chicken was confirmed in a previous study (Halevy et al. 2004).

The frequency of SCs, which is the number of SC nuclei divided by the sum of myonuclei and SC nuclei, has been used to study the distribution of SCs in skeletal muscles (Schmalbruch and Helhammer 1977; Gibson and Schultz 1983; Hikida et al. 1998; Roth et al. 2000; Sajko et al. 2002). This frequency of SCs was found to vary among species (Castillo de Maruenda and Franzini-Armstrong 1978; Novotova and Uhrik 1992) and muscles with different fiber types (Schmalbruch and Hellhammer 1977; Schultz and McCormick 1994). However, the trend in temporal distribution for most species and muscles studied showed a decrease in SC number and frequency

with age. For example, while SCs comprise about 30% of muscle nuclei in newborn rodents, this percent decreases to about 4% in adults and 2% in senile animals (Snow 1977; Gibson and Schultz 1983; Hawke and Garry 2001). Many questions remain concerning the spatial distribution of SCs within skeletal muscles. Previous studies have shown that SCs tend to be more concentrated near specific anatomic structures, such as blood capillaries and motor end plates (Schultz and McCormick 1994). Along the rest of the length of individual muscle fibers, SCs have been described as either evenly (Snow 1981) or randomly (Novotova and Uhrik 1992) distributed.

The ends of skeletal muscle fibers have been shown to be comparatively active sites of postnatal growth. New sarcomeres are added in series to the ends of existing myofibrils during normal longitudinal growth of the muscles of mammals (Goldspink 2003) and fish (Ennion et al. 1999). In mouse muscles in which fibers are arranged in series, longitudinal growth of the fibers also produces an increase in the diameter of the muscle (Paul and Rosenthal 2002). Nuclei are preferentially added to the ends of growing myotubes in rats (Zhang and Mclennan 1995). Rosser et al. (2002) have previously shown that myonuclei are more concentrated at the ends of maturing fibers of post-hatch chicken pectoralis. In experimentally induced lengthening of immature leg muscle of rabbits, a greater proportion of SCs expressing proliferating cell nuclear antigen were located at the musculotendinous junction than in other regions of the muscle (Tsujimura et al. 2006).

The pectoralis muscle of the chicken is composed of fibers that overlap one another in series from the origin to the insertion of the muscle (Gaunt and Gans 1992, Trotter 1993). Consequently, a transverse section anywhere through the belly of the muscle will include many fiber ends (Rosser et al. 1995, 2000, 2002). Distinct myosin heavy chain (MyHC) isoforms are sequentially expressed within the fibers of developing chicken pectoralis (Bandman and Rosser 2000). Embryonic isoforms are supplanted by a neonatal isoform that is in turn replaced by an adult isoform. In each muscle fiber, replacement of neonatal by adult MyHC isoform progresses from the centrally located motor end plate towards the tapered ends of the fiber (Rosser et al. 2000). Fiber ends invariably have the smaller diameters, and retain densities of neonatal myosin comparable to those observed in neonatal muscle (Rosser et al. 1995, 2000, 2002).

This study elucidates the temporal and spatial patterns of SC distribution in the post-hatch chicken pectoralis. It tests the hypothesis that there is a greater frequency and concentration of SCs at the ends of muscle fibers. The same chicken pectoralis samples used in the previous studies of Rosser et al. (2000, 2002) are examined. Using immunocytochemical techniques that include an antibody against Pax7, SCs are detected among different regions of the muscle fibers during maturation. While the frequency of SCs is significantly reduced with age, it remains high at the ends of the fibers where SCs are also located closer to one another.

3.2 Materials and Methods

3.2.1 Experimental Model

Female White Leghorn chickens (*Gallus gallus*; Hy-Line W-36, Clark Hy-Line, Brandon, Manitoba) were hatched at the same time and raised under identical conditions at the University of Saskatchewan, Department of Animal and Poultry Science as described previously in Rosser et al. (2002). It has already been established that chickens reach sexual maturity around the age of 150 days post-hatch (Limburg 1975). Following the Canadian Council on Animal Care Guidelines, and with the approval of the University of Saskatchewan Committee on Animal Care and Supply, three birds were killed by cervical dislocation at each of the following ages; 9, 23, 30, 49, 62, 79 and 115 days post-hatch.

3.2.2 Tissue Preparation and Sectioning

Three muscle samples were excised from the cranial half of the superficial region of the main part of the left pectoralis muscle (M. pectoralis pars sternobrachialis; Vanden Berge and Zweers 1993) of each bird. Each sample was approximately 0.5 x 0.5 x 1-2 cm. The long axis of each sample was parallel to the direction of the muscle fibers. Each sample was then coated with O.C.T. compound (Miles Inc., Indiana, USA) and immediately frozen in 2-methylbutane cooled via liquid nitrogen (Sewry and Dubowitz 2001). After that, samples were stored at -80°C.

Serial cross-sections of 10 μm thickness were cut at -20°C using a cryostat. Two serial sections were picked up on each ProbeOn Plus microscopic slide (Fisher Scientific Ltd., Ontario, Canada). Serial slides bearing sections were numbered and stored at -20°C. Along with these cross sections, some longitudinal sections were obtained in order to measure the length of SC nuclei.

3.2.3 Antibodies and Nuclear Labeling Agent

The previously characterized primary antibodies used for immunostaining were anti-Pax7, anti-laminin and anti-neonatal myosin heavy chain. All were diluted in blocking solution (see following). Anti-Pax7 (Developmental Studies Hybridoma Bank, Iowa, USA; Berggren et al. 2001), a mouse monoclonal developed against chicken Pax7, was used at a dilution of 1:100 to detect SC nuclei. Anti-laminin (L9393, Sigma Chemical Co., Missouri, USA; Tisay and Key 1999) a rabbit polyclonal raised against the glycoprotein laminin of mice, was used at 1:200 to detect the basal lamina of skeletal muscle fibers. The anti-neonatal MyHC (2E9, gift from Dr. E. Bandman), a mouse monoclonal developed against chicken neonatal MyHC isoform (Bandman 1985; Moore et al. 1992), was used at 1:500. Depending on the density of labeling by the anti-neonatal MyHC antibody, it was possible to distinguish three different fiber profile types; neonatal, transforming and adult (see following).

Fluorescein isothiocyanate-conjugated anti-mouse and tetramethyl rhodamine anti-rabbit secondary antibodies (A-11010 and A-11001, Molecular Probes, Oregon,

USA) were used to detect, respectively, anti-Pax7 (green) and anti-laminin (red) when viewed with epifluorescent microscopy. Each of these secondary antibodies was prepared at a dilution of 1:200 in phosphate-buffered saline (PBS; 0.02 M sodium phosphate buffer, 0.15 M sodium chloride, pH 7.2). Biotin-conjugated anti-mouse secondary antibody (B-9904, Sigma Chemical Co., Missouri, USA) was used at a dilution of 1:500 in another set of experiments to visualize labeling of neonatal myosin by the anti-neonatal MyHC antibody when viewed under bright-field microscopy. Hoechst 33258 (Bisbenzimide, 10 mg/ml stock concentration, Sigma Chemical Co., Missouri, USA) was applied at a dilution of 1:1,500,000 in PBS to label the DNA in nuclei (blue) under epifluorescent microscopy.

3.2.4 Immunocytochemical Techniques

Serial numbered slides were removed from the freezer and air dried for 15 minutes. Sections were treated for 30 minutes with at least 200 µl of blocking solution, which consisted of 5mM ethylenediaminetetraacetic acid (EDTA) in PBS, 5% goat serum and 1% bovine serum albumin. Blocking solution was drained from each slide onto a paper towel.

Slides were then alternatively treated either with the anti-neonatal MyHC antibody for bright-field microscopy or with anti-Pax7, anti-laminin and Hoechst for epifluorescent microscopy. Using this approach the type of fiber profile (neonatal, transforming or adult) was identified on one slide, while SC nuclei and myonuclei within the same fiber were located in the serial sections on the adjacent slide.

3.2.4.1 Bright-Field Immunocytochemistry

The anti-neonatal MyHC was applied over the sections using 150 µl on each slide for 70 minutes at room temperature. Slides were then washed twice in fresh PBS solution for five minutes per wash. The biotinylated goat anti-mouse secondary antibody was then applied over the sections on each slide for 30 minutes at room temperature, followed by two five minutes washes in fresh PBS solution.

Slides were then placed in 3% hydrogen peroxide in methanol for five minutes followed by two five minutes washes in PBS. Next 150 µl of Avidin ABC kit (Vector Laboratories Inc., California, USA) diluted in PBS was applied over the sections that were then kept in the dark for 60 minutes. Slides were then washed in phosphate buffer solution (PB; 0.02 M sodium phosphate buffer, pH 7.2) twice for five minutes each. To obtain a specific color reaction visible under the bright-field microscope, slides were immersed in 0.05% diaminobenzidine in PB for five minutes. This was followed by a five minutes wash in PB, and then a five minute wash in PBS.

Slides were then fixed with 4% formaldehyde in PBS solution for three minutes, and then washed for five minutes in fresh PBS. After that, slides were rinsed for five minutes under tap water. Finally, the wet slides were mounted with cover slips using Geltol mounting medium (Thermo Shandon, Pennsylvania, USA) and left for 20 minutes to harden before being examined under the microscope.

3.2.4.2 Epifluorescent Immunocytochemistry

Anti-Pax7 and anti-laminin primary antibodies were diluted in blocking solution and applied together over the sections on slides using 150 µl per slide. The slides were kept overnight in the dark at 4°C. Next morning, slides were washed three times in fresh PBS solution for five minutes per wash. A cocktail containing the fluorescein and tetramethyl secondary antibodies A-11010 and A11001 was then applied over the sections on each slide for 30 minutes at room temperature. This was followed by two five minutes washes in fresh PBS solution. Hoechst 33258 diluted 1:1,500,000 in PBS was applied over the sections for five minutes, followed by two additional five minutes washes in PBS. Slides were then fixed with 4% formaldehyde in PBS, and mounted in Geltol as described in the preceding section.

3.2.5 Image Analysis and Data Collection

Three fields of view, each having entirely different fiber profiles, were captured from each immunofluorescent slide using a Zeiss Axioskop 20 microscope (Carl Zeiss GmbH, Jena, Germany) equipped for epifluorescence and a Sony S70 (Sony Corporation, Tokyo, Japan) digital still camera. Three epifluorescent images, each viewed through a different wavelength filter, were acquired from each field. The resultant images were all nuclei in blue, SC nuclei in green and basal laminae in red (Fig. 1). Images were subsequently transferred to a Macintosh G4 computer (Apple Computers Inc., California, USA) and the three images for each field of view were superimposed using Adobe Photoshop (Adobe System Inc., California, USA).

Images of the anti-neonatal MyHC antibody labeled slides were taken under bright-field microscopy from sections serial to those used for epifluorescence, and included the same fields of view that were photographed under epifluorescence (Fig. 1). These bright-field images were then transferred to the computer and, using a graphic converter program (Lemke Software GmbH, Peine, Germany), image format was changed to TIFF format. Scion imaging program (developed by US National Institute of Health; Image J is the current version that is available on the internet at http://rsb.info.nih.gov/ij) was then used to set the images to gray scale for measuring the optical density and the ellipse minor axis of individual fibers.

The optical density and ellipse minor axis of 200 contiguous fiber profiles from each animal were measured. Optical density measurements were used to classify fiber profiles as neonatal, transforming or adult. White and black were automatically assigned optical density values of 0 and 255, respectively, at the ends of an arithmetic linear regression ($r^2 = 1.00$). Pectoralis tissue from 9 day old chicks was used as a neonatal density control, since it contains only neonatal myosin (Bandman and Rosser 2000). Larger fiber profiles of pectoralis tissue from 115 day old chickens were used as a control for the absence of neonatal myosin (Rosser et al. 2000 and 2002). All fiber profiles quantified at age 9 days had optical densities of >110, while control adult profiles at age 115 days had values of <80. Thus, in all sections studied fiber profiles with a density of >110 were classified as neonatal, profiles <80 were classified as adult, and profiles ≥80 and ≤110 were classified as transforming. Ellipse minor axis was used to assess the size of fiber cross sectional

profiles from the pectoralis of birds aged 9, 49 and 62 days. These fiber profiles were then arranged into groups according to size by 10 μm increments. Rosser et al. (2000) have previously shown that ellipse minor axis is identical to lesser fiber diameter, which was defined as the maximum aspect across the lesser aspect of a fiber. Lesser fiber diameter is used to overcome distortion that may result when a muscle fiber is cut obliquely, rather than transversely (Dubowitz 1985).

The numbers of satellite cell nuclei (SCN) and myonuclei (MN) within each of the 200 fibers of each animal were totaled. After that, the frequency of SCs was calculated for each animal, for each fiber profile type and for each fiber size range group using the formula that frequency = (SCN / SCN+MN) X 100% (Schmalbruch and Helhammer 1977). The mean values for SC frequency per fiber type and size group were obtained from three animals at each age.

The lengths of SC nuclei were measured from the longitudinal sections obtained from three animals at each of the following ages; 9, 30, 49, 62 and 115 days. Applying the same protocol used to label Pax7 within the cross sections, the lengths of 15-22 SC nuclei from each animal were measured using the Scion imaging program. The average length of SC nuclei was calculated for each age, and the mean value of the averages was determined.

The number of SCs per unit length of fiber was calculated for each type of fiber profile (neonatal, transforming and adult) using a formula from Castillo de Maruenda and Franzini-Armstrong (1978). This formula is N= A/(Ln + M), where N is the number of SCs per unit length of fiber, A is the mean number of SC nuclei per fiber profile, Ln is the average length of SC nucleus and M is the thickness of tissue section. The surface area of the plasmalemma per unit length of the fiber was also measured for different profiles using the formula S= πEU, where S is the surface area of the plasmalemma per unit length of fiber, E is the ellipse minor axis and U is the unit length of the fiber (1 mm). Lastly, the area of muscle fiber plasmalemma per each SC was determined by dividing the surface area measurements (S) by the number of SCs (N) in each type of fiber profile.

3.3 Statistics

After determining the homogeneity of variance, data were evaluated by one-way and univariate analysis of variance (ANOVA) at 5% level of significance. The samples were blocked into different age groups and randomized complete block design analysis was applied. If a significant ($P < 0.05$) difference was found, Scheffe's post hoc analysis test was applied to examine statistical differences between groups. Kruskal-Wallis non-parametric test was used to evaluate surface area per SC data, and t-test for two independent samples was further applied to compare the neonatal group along with transforming or adult group. All statistical tests were performed using SPSS program (standard version 12.0.0, SPSS Inc., Illinois, USA).

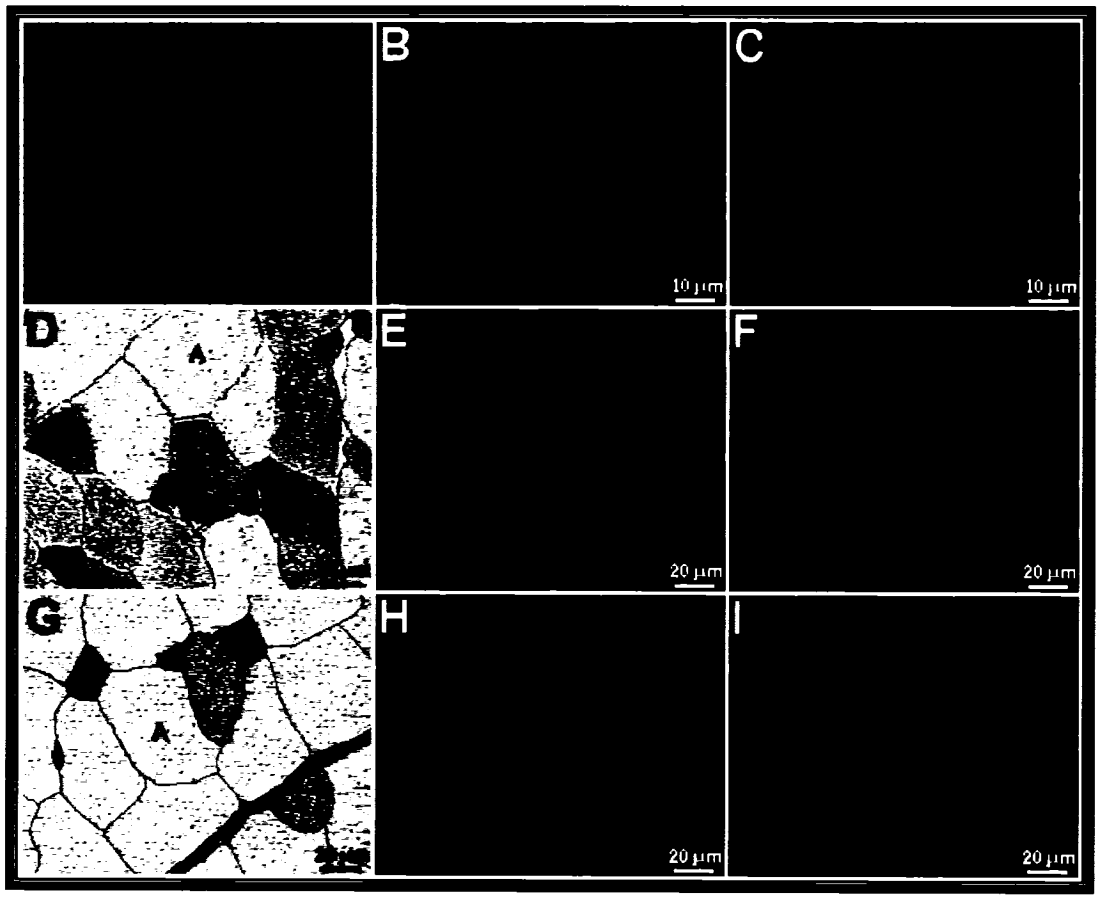

Figure 1: Immunocytochemical labeling of transverse serial sections of chicken pectoralis. Chicken pectoralis aged 9 (**A**, **B**, **C**), 49 (**D**, **E**, **F**) and 79 (**G**, **H**, **I**) days post hatch. **A**, **D** and **G** are viewed by bright-field and labeled by the anti-neonatal MyHC antibody to distinguish the type of fiber profile (neonatal=N, transforming=T and adult=A). **B**, **E** and **H** are, respectively, sections cut serial to **A**, **D** and **G** viewed under epifluorescence. All nuclei appear blue due to labeling by Hoechst, and basal laminae red with labeling by anti-laminin. **C**, **F** and **I** are the same sections as **B**, **E** and **H**, respectively, showing satellite cell nuclei in green labeled by anti-Pax7 and the basal laminae in red labeled by anti-laminin.

3.4 Results

3.4.1 Expression of Pax7 by SC nuclei

The locations of Pax7 labeled (+) nuclei relative to basal laminae were determined from three animals at each of four different developmental ages; 9, 49, 62 and 115 days post hatch (Table 1). It was found that about 97-98% of the Pax7+ nuclei were located beneath the basal lamina. Consequently, these nuclei were classified as SC nuclei. The remaining 2-3% were nuclei located outside of the basal lamina in the surrounding endomysium and perimysium (Fig. 2).

3.4.2 Length of SC Nuclei

Mean length of SC nuclei was determined on longitudinal sections obtained from three animals at each of the following ages; 9, 30, 49, 62, 115 days post hatch (Table 2). There were no significant ($P > 0.05$) differences in the mean length of SC nuclei either within or among these ages. Thus, there is a uniform length for SC nuclei in chicken pectoralis during development. The overall mean length was 10.28 μm ± 0.10 (± standard deviation).

Age (days)	9	49	62	115
Number of fibers	1,500	1,680	1,650	1,485
Pax7+ nuclei within basal laminae	159	161	144	103
Pax7+ nuclei outside basal laminae	3	5	4	2
% of Pax7+ SC nuclei	98.15%	96.99%	97.30%	98.10%
% of Pax7+ non-SC nuclei	1.85%	3.01%	2.70%	1.90%

Table 1: Percent of Pax7+ nuclei that are satellite cell (SC) nuclei. "Number of fibers" for each age listed represents the total number of fibers analyzed from three birds. Approximately 500 fibers per animal were analyzed at each age. The percent of Pax7+ nuclei that are SC nuclei (located beneath the basal laminae) was calculated. At each age a small percentage of the Pax7+ nuclei were found in locations not characteristic of SCs.

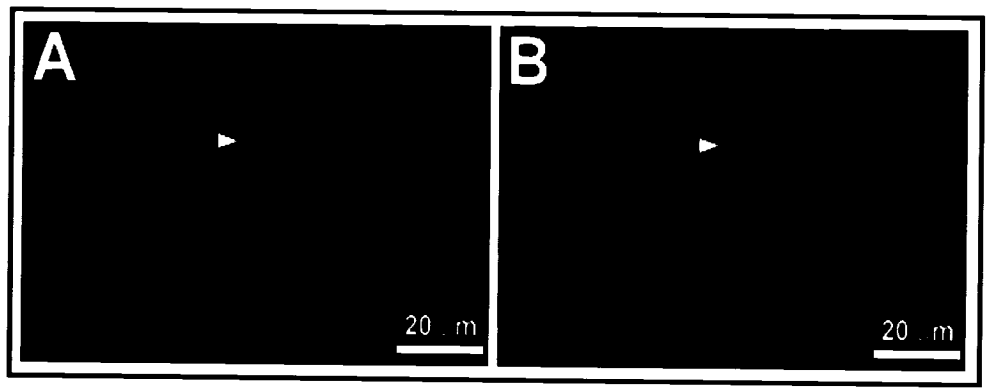

Figure 2: Pax7+ nucleus outside of the basal laminae. A cross section prepared from pectoralis muscle of a 49 day old chicken and labeled for immunofluorescence by Hoechst in blue (**A**), anti-Pax7 in green (**B**) and anti-laminin in red (**A** and **B**). The arrowheads indicate a Pax7+ nucleus that is located outside of the fibers' basal laminae within the interstitial tissue of the muscle.

Age (days)	9	30	49	62	115
Mean Length of SC nuclei (μm) ± SE	10.30 ± 0.06	10.17 ± 0.03	10.33 ± 0.07	10.40 ± 0.06	10.18 ± 0.09

Table 2: Length of satellite cell (SC) nuclei. Longitudinal sections were cut from three birds at each of the five ages. The lengths of 15 to 22 SC nuclei were measured from each bird. Each value represents the average of the means from three birds at each age ± standard error (SE). There was no significant ($P > 0.05$) difference in the lengths of SC nuclei among the different aged birds studied. The mean length of SC nuclei in the five ages was found to be 10.28 μm ± 0.06 (SE).

3.4.3 Frequency of SCs with Age

The mean frequency of SCs decreased significantly ($P < 0.01$) with age. As illustrated in Figure 3, the frequency declined from 20.04% at age 9 days to 4.86% at 115 days post hatch. These findings were consistent with previous results of Halevy et al. (2004), and reflect observations from studies of other species (Hawke and Garry 2001). Furthermore, this data showed that the declining pattern of SC frequency with age is a curvilinear regression (SC frequency= 34.129-6.091 Ln [age], r^2= 0.953).

3.4.4 Frequency of SCs with Type of Fiber Profile

By correlating the frequency of SCs to the type of fiber profile (neonatal, transforming or adult) it was found that the highest frequency was among the neonatal profiles. SC frequency in neonatal profiles ranged from 13.65% to 20.04% during development. The frequency of SCs was dramatically ($P < 0.001$) decreased with repression of neonatal MyHC isoform expression (Fig. 4). There was no significant difference ($P > 0.05$) in SC frequency between transforming and adult profiles. Overall, the mean frequency of SCs in transforming and adult profiles never reached 10%. All fiber profiles at ages 9 and 23 days were neonatal, while most of the profiles at age 115 days were adult. The transforming profiles appear somewhere between 23 and 30 days of age (see Rosser et al. 2000). These findings support the current study's hypothesis that there is a greater frequency of SCs at the ends of muscle fibers, as after 23 days of age neonatal profiles are in fact the tapered ends of the fibers (Rosser et al. 2000).

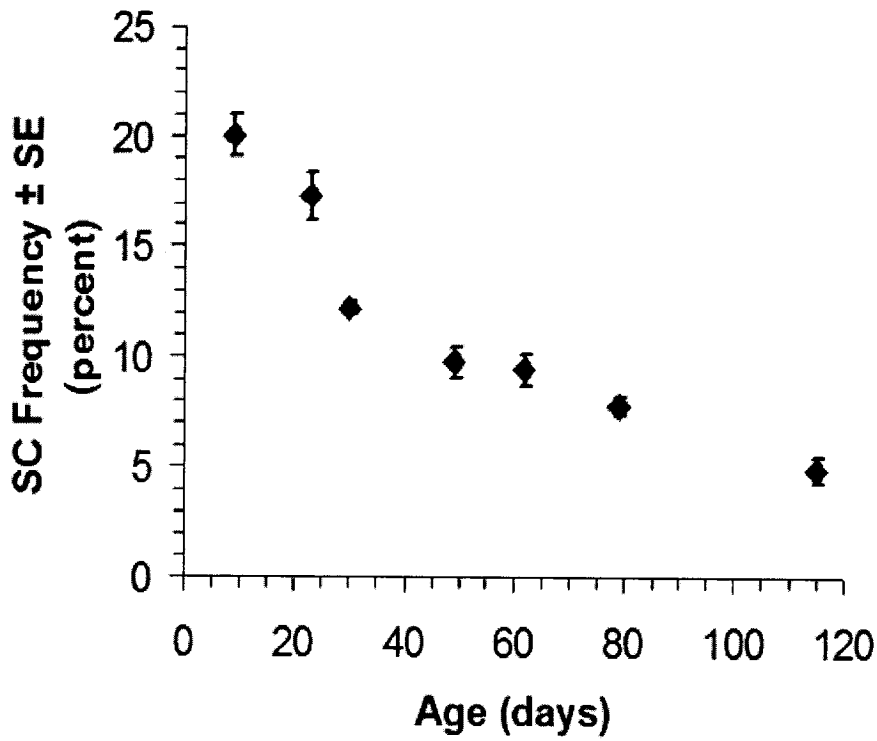

Figure 3: Satellite cell (SC) frequency during development. The frequency of SCs, (SCN/SCN+MN)x100%, in pectoralis muscle of the chicken was determined for three birds at each of the following ages; 9, 23, 30, 49, 62, 79 and 115 days post hatch. Numbers of SC nuclei (SCN) and myonuclei (MN) were counted separately for 200 contiguous fibers per each muscle sample. Each symbol represents the mean SC frequency (%) ± standard error (SE). The percentage of SC frequency is significantly (one-way ANOVA, $P < 0.01$) decreased with age.

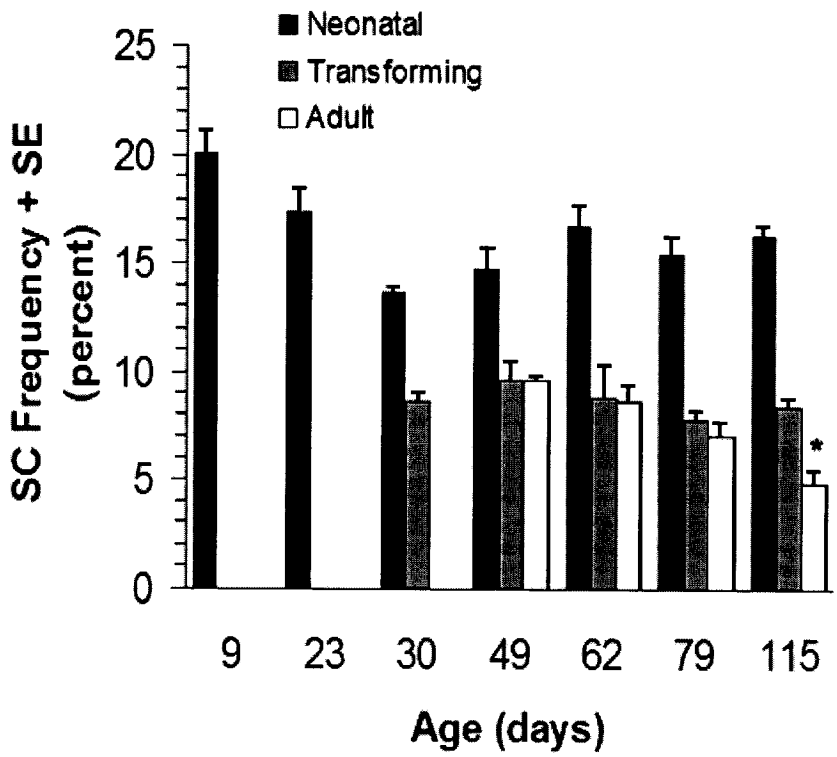

Figure 4: Satellite cell (SC) frequency during development in neonatal, transforming and adult fiber profiles. Each column represents mean SC frequency (%) + standard error (SE). The frequency is significantly ($P < 0.001$) greater in neonatal profiles than in either transforming or adult profiles at all ages examined. After 23 days of age, the neonatal profiles are the ends of more mature transforming and adult profiles (Rosser et al. 2000). * At age 115 days the frequency of SCs within adult profiles is significantly ($P < 0.05$) lower than the frequency within transforming profiles and adult profiles at all ages.

3.4.5 Frequency of SCs with Size of Fiber Profile

The mean frequency of SCs decreased with increasing fiber size. The ellipse minor axis of the largest profiles at age 9 days did not exceed 20 μm. At this age although fibers ranging from 0-10 μm had a greater frequency of SCs than those ranging from 10.01-20 μm, this difference was not statistically significant ($P = 0.12$, Fig. 5A). At age 49 days and 62 days there was a significant ($P < 0.01$) decrease in the mean frequency of SCs as the ellipse minor axis increased (Fig. 5B, C). This decrease was most prominent as the ellipse minor axis increased from the range of 0.0-10.00 μm to 10.01-20 μm. These findings strongly support the hypothesis that the tapered fiber ends have a higher frequency of SCs, since the smaller fiber profiles seen at the age of 49 and 62 days are the tapered ends of muscle fibers (see Rosser et al. 2000).

3.4.6 Surface Area of Sarcolemma per SC

The surface area of muscle fiber sarcolemma per SC was calculated for each different type of fiber profile at each of the following ages; 9, 49, 62, 79, 115 days post hatch. There was less area of sarcolemma per SC in neonatal profiles than in other profiles (Fig. 6). The difference was statistically significant ($P < 0.05$) between neonatal and either transforming or adult profiles, but not between transforming and adult profiles ($P = 0.38$). These results show that there is a greater concentration of SCs at fiber ends, represented by neonatal profiles, than in other regions of the muscle fibers.

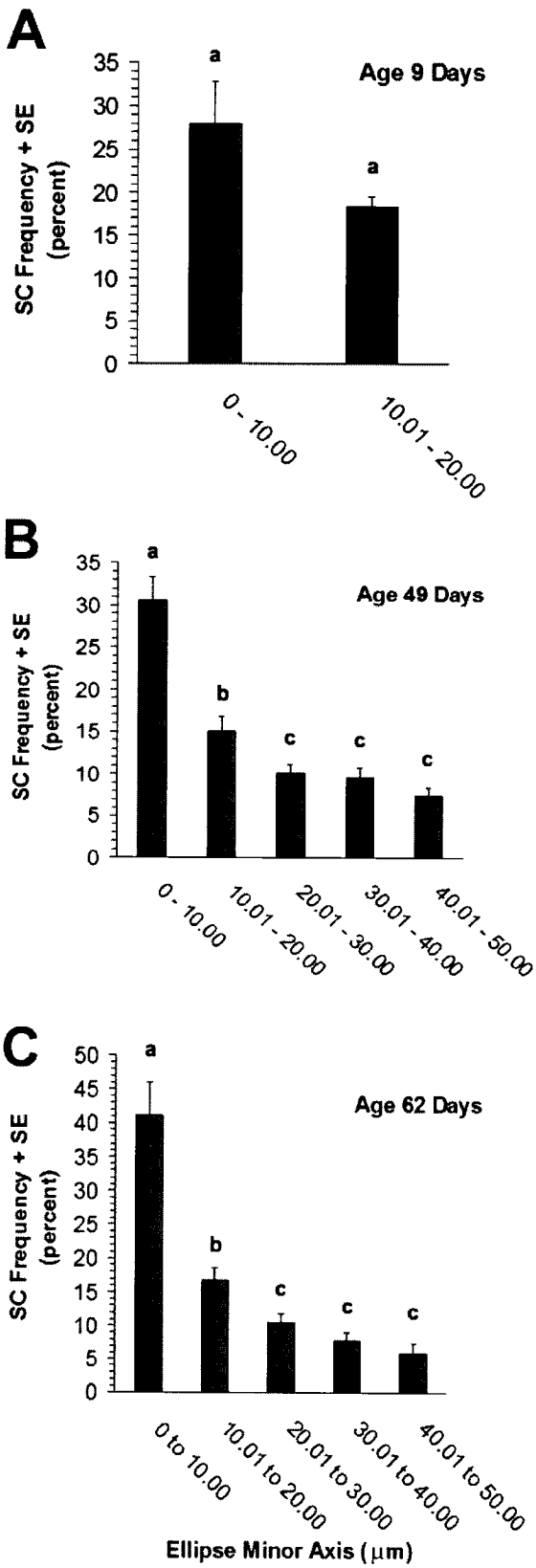

Figure 5: Correlation of satellite cell frequency with fiber size. Three birds were studied at three different ages; (**A**) 9 days, (**B**) 49 days and (**C**) 62 days. Between 200-250 fiber cross sections from each pectoralis studied were arranged into different groups according to their ellipse minor axes values. Each column represents mean SC frequency (%) + standard error (SE). The frequency of SCs in ages 49 and 62 days is significantly ($P < 0.01$) reduced with increasing fiber size. The smaller fiber profiles are the tapered ends of larger profiles (Rosser et al. 2000). In each age graph, columns with different letters are statistically different (Scheffe's, $P < 0.05$).

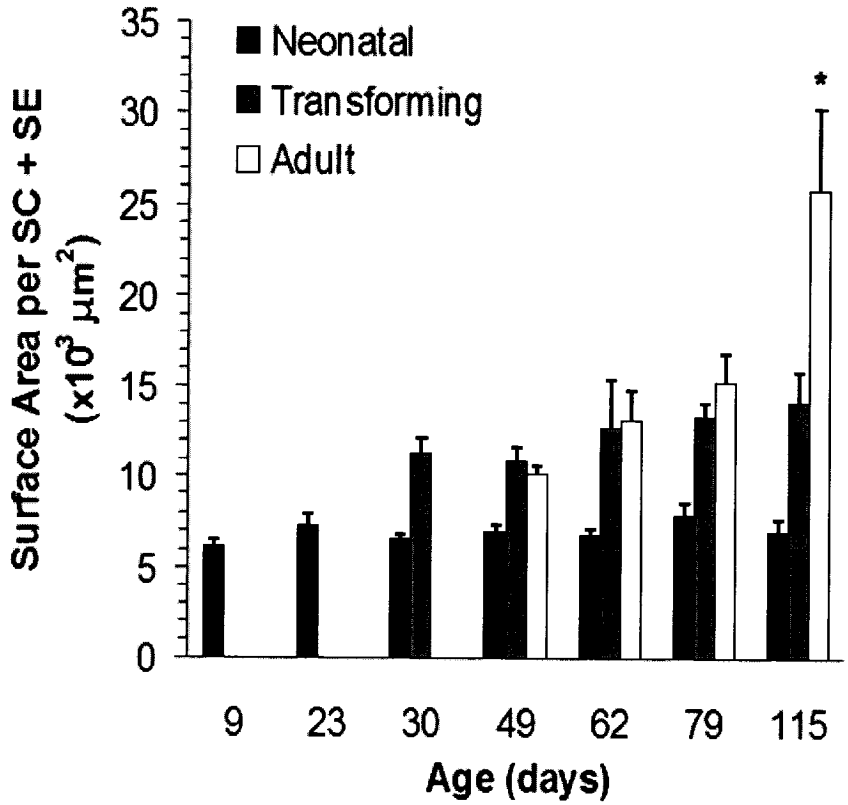

Figure 6: Correlation of surface area of sarcolemma per satellite cell (SC) with different types of fiber profiles during development. Each column represents mean surface area of sarcolemma x 10^3 μm^2 + standard error (SE). There is significantly ($P < 0.05$) less surface area per SC in neonatal profiles than transforming or adult profiles. * At age 115 days, the surface area per SC in adult profiles is significantly ($P < 0.05$) larger than that in transforming profiles and adult profiles at all ages.

3.5 Discussion

This study reveals new information about the temporal and spatial patterns of SC distribution. It is the first work to demonstrate that throughout growth and maturation there is both a greater frequency and a higher concentration of SCs at the terminal tips of skeletal muscle fibers. Earlier works showed that the ends of maturing skeletal muscle fibers in the post-hatch pectoralis of the chicken (Rosser et al. 2000) and pigeon (Bartnik et al. 1999) retain high densities of neonatal MyHC isoform. The current study finds that while SC frequency decreases during development with repression of neonatal MyHC isoform expression along the fiber lengths, the tapered fiber ends retaining neonatal myosin also keep a high frequency of SCs. To further validate these results the frequencies of SCs for different sizes of fiber profiles were obtained, and a higher frequency of SCs was found in smaller fiber profiles. This corroborates the results correlating greater SC frequency with the expression of neonatal myosin, as the smaller fiber profiles are the tapered ends of skeletal muscle fibers (Rosser et al. 2000). A greater concentration of SCs at the ends of skeletal muscle fibers was also observed, as shown by significantly less area of plasmalemma per SC in those fiber regions expressing neonatal myosin.

Developing skeletal muscle fibers grow in both length and width (Zhang and McLennan 1995; Paul and Rosenthal 2002). It has been established that the terminal ends of maturing muscle fibers are the regions for longitudinal growth within the muscle (Swatland 1994; Goldspink 2003). Earlier work showed that smaller myonuclear domains are present at the tapered ends of the fibers in the post-hatch

chicken pectoralis (Rosser et al. 2002). Myonuclear domain is the volume of sarcoplasm associated with a single myonucleus, and each myonucleus is responsible for protein synthesis within its domain (Hall and Ralston 1989; Allen et al. 1999). Smaller myonuclear domain can be an indication of greater protein synthesis, since myonuclei tend to be more concentrated in regions of active protein synthesis (Edgerton and Roy 1991; Winchester and Gonyea 1992; Allen et al. 1999; Ohira et al. 2001). Myonuclei arise from SCs (Seale and Rudnicki 2000; Hawke and Garry 2001). Presumably the higher frequency and concentration of SCs now observed at the fiber ends is correlated with the demand for more myonuclei there, due to greater protein synthesis required for longitudinal growth. Nevertheless, further study is required to determine whether SCs at the fiber ends are quiescent or active. A study of mouse fast and slow-twitch muscles has revealed that while SC numbers fall with age, their myogenic potential does not (Shefer et al. 2006). Indeed, SCs from mature fibers were shown to retain the ability to give rise to non-myogenic descendents (Asakura et al. 2001, Shefer et al. 2004). Thus, whether they are quiescent or active, SCs at the ends of maturing fibers in the present study should retain their myogenic potential.

SC nuclei have a uniform average length of approximately 10.28 μm throughout maturation of the post-hatch pectoralis. This length is comparable to those found in previous studies of other species (Muir et al. 1970, Schmalbruch 1977). Earlier studies of other species and muscles found that SCs were slightly shorter than myonuclei (Schultz and McCormick 1994). While this generalization

appears to hold for this study, the length of SC nuclei in the chicken pectoralis is not statistically (P = 0.55) different from the mean length of myonuclei (10.58 ± 0.08 μm; mean ± SD) obtained from the same samples (Rosser et al. 2002). Since SC nuclei become myonuclei after their differentiation and fusion to muscle fibers (Seale and Rudnicki 2000; Hawke and Garry 2001, Wozniak et al. 2005), it is reasonable to expect a similar nuclear length between SC nuclei and myonuclei. This uniform nuclear length does not mean that there will be lack of variation in cell size or nuclear condition during the different stages of the cells (McCormick and Schultz 1994; Anderson 2000). For example, a significant increase in SC cytoplasmic area compared with nuclear area was found during SC activation (Sinha-Hikim et al. 2003).

Pax7 is a common denominator in muscle stem cells (McKinnell et al. 2005). Since Pax7 is expressed in SCs during their different stages of quiescence, proliferation and differentiation (Seale et al. 2000; Halevy et al. 2004), this study depends upon Pax7 transcription factor for identification of all SC subpopulations. About 97-98% of Pax7+ cells are localized beneath fiber basal laminae in positions characteristic for SCs. Hence, it is inferred that these Pax7+ nuclei are SC nuclei. As the remaining 2-3% Pax7+ cells are located in the interstitial spaces of the muscle outside of the basal laminae, they are not included in the calculations for SCs.

Infrequent myogenic stem cells residing outside of the muscle fiber basement membrane have been identified in various studies (Tamaki et al. 2002; Dreyfus 2004;

Collins 2006). The frequency of such cells is low and, thus, correlates with the distribution of interstitial Pax7+ cells observed in the present study. The interstitial cells may be SCs in transition from one myofiber to another, as has been previously reported (Hughes and Blau 1990). They could also be comparable to the progenitors of nascent myofibers, as has been described in chicken muscle subjected to weight overload (McCormick and Schultz 1992) or rapidly growing rat plantaris muscle (Tamaki et al. 2002). However, earlier work has shown that small fiber profiles expressing neonatal myosin in maturing chicken pectoralis represent the tapered ends of larger fiber profiles and not nascent fibers (Rosser et al 2000).

Mononucleate bone marrow derived cells can become muscle SCs (Fukada et al. 2002, LaBarge and Blau 2002). These bone marrow derived cells can contribute to the growth and repair of muscle fibers in response to injury (Dreyfus et al. 2004) and normal physiologic stress (Palmero et al. 2005). Some of these Bone Marrow Derived Cells are of hematopoietic origin (Camargo et al. 2003, Corbel et al 2003). It has, however, been suggested that identification of the optimal hematopoietic stem cell for muscle regeneration remains elusive, and that these cells are unlikely to play a significant role under normal conditions (Sherwood et al. 2004, McKinnell et al. 2005). As bone marrow derived cells do not appear to express Pax7 (Asakura et al. 2002, Collins 2006), in the present study it is unlikely that the Pax7+ nuclei found outside of muscle fiber basal laminae are bone marrow derived cells. Also, as has been observed by studies of rat (Gavazzi et al. 1995) and a diversity of other species (Sigma Chemical Co.), in the present study laminin within the walls of blood vessels

was labeled by the anti-laminin antibody used to recognize muscle basal laminae. In no instance was a Pax7+ interstitial cell ever within a blood vessel, nor did any of these cells appear to be associated with blood vessels.

In summary, this study shows that SC frequency is significantly decreased during maturation of the chicken skeletal muscle. This indicates a reduced ability of the muscle to grow and regenerate at older ages. The study also reveals a higher frequency and a greater concentration of SCs at the tapered ends of growing skeletal muscle fibers. This indicates a major contribution of these cells in the longitudinal growth of muscle fibers, since new sarcomeres are added to the fiber ends during longitudinal growth.

4.0 CHAPTER

THE IN VIVO EXPRESSION PATTERNS OF MYOD AND MYOGENIN REGULATORY FACTORS BY SATELLITE CELL NUCLEI AND MYONUCLEI

4.1 Introduction

Satellite cells (SCs) are mononuclear myogenic stem cells, located between the basal lamina and plasmalemma of the skeletal muscle fiber (Mauro 1961; Mauir et al. 1965; Muir 1970; Mckinnell et al. 2005). They were discovered in 1961 through the use of electron microscopy (Mauro 1961; Katz 1961). SCs are highly active during postnatal development, where they contribute to muscle growth and regeneration by fusing to muscle fibers where their nuclei become new myonuclei (Schultz et al. 1978; Wozniak et al. 2005). In normal mature muscle, SCs are usually quiescent with relatively reduced cytoplasmic volume and organelles (Schultz et al. 1978; Hawke and Garry 2001; Wozniak et al. 2005). Under proper stimulation, such as an injury to a muscle fiber or exercise, SCs become active, enter the cell cycle and start to proliferate. Afterwards, most of the proliferated SCs start to differentiate by either fusing to a preexisting muscle fiber or, to a lesser extent, fusing together to form a new fiber (Bischoff 1994; Seale and Rudnicki 2000; Hawke and Garry 2001; Mckinnell 2005). The remaining proliferating SCs return back to quiescence and maintain a reservoir of SCs.

Identification of SCs can be made based upon their characteristic location beneath the basal lamina either by using electron microscopy or immunocytochemistry. Immunocytochemistry is more commonly used, since it is easier to apply than electron microscopy. Also, SCs can be identified by detecting specific proteins expressed only by them such as the paired box transcription factor 7 (Pax7), M-Cadherin or CD 34 (Hawke and Garry 2001; Morgan and Partridge 2003).

The advantage of Pax7 is that it is expressed by SCs during all their stages: quiescent, proliferative and differentiated (Seale et al. 2000; Olguin and Olwin 2004; Relaix et al. 2005; Kuang et al. 2006). Pax7 was found to be specifically expressed by SCs in rodents (Seale et al. 2000). Similarly, the specific expression of Pax7 by all SCs in the pectoralis muscle of post-hatch chickens was confirmed in the previous study in chapter 3.0.

MyoD and Myogenin are members of a specific skeletal-muscle transcription factor family called the myogenic regulatory factors (Funk et al. 1991; Megeney and Rudnicki 1995). This family is composed of several members including MyoD, Myogenin, MRF4 and Myf5. MyoD and Myogenin are considered the main members of this family, while Myf5 and MRF4 can carry out similar functions to MyoD and Myogenin respectively (Hawke and Garry 2001). Myogenic regulatory factors are basic helix-loop-helix proteins that are critical for establishing the myogenic lineage during embryogenesis, and for controlling the terminal differentiation of myoblasts into myotubes (Ludolph and Konieczny 1995; Hawke and Garry 2001; Tapscott 2005). These factors control the expression of many muscle genes during embryonic and postnatal developmental stages, such as muscle structural and contractile proteins, nicotinic acetylcholine receptors and specific muscle enzymes such as creatine kinase (Nicolas et al. 1996; Hawke and Garry 2001).

Several studies of MyoD and Myogenin expression in cultured myoblasts have suggested that these factors fulfill distinct roles in myogenesis and regeneration

(Sorrentino et al. 1990; Montarras et al. 1991; Cornelison and Wold 1997; Yablonka-Reuveni et al. 1999; Cornelison et al. 2000; Yablonka-Reuveni and Paterson 2001). These *in vitro* studies reveal that MyoD expression always precedes the expression of Myogenin. They have suggested a role for MyoD at the early stages of myoblast activation, while Myogenin expression is restricted to the late differentiation stage of cultured myoblasts (Montarras et al. 1991; Seale and Rudnicki 2000; Yablonka-Reuveni and Paterson 2001). Until now, few studies have reported the expression of MyoD and Myogenin *in vivo* by either myonuclei or satellite cell nuclei (Dedkov et al. 2003; Ishido et al. 2004a; 2004b). However, these previous studies did not attempt to discern the patterns of expression for these factors throughout postnatal development.

The present study tests the hypothesis that MyoD and Myogenin transcription factors have distinctive patterns of expression within SC nuclei during maturation. The chicken pectoralis muscle was the experimental model used in this study. Two sets of immunofluorescence experiments were applied. Each set included antibody against Pax7, to identify SC nuclei, and either an anti-MyoD or an anti-Myogenin antibody. With maturation, significantly increased numbers of myonuclei and SC nuclei are found to express MyoD (MyoD+) until adulthood, where the numbers of these MyoD+ nuclei start to decline. On the other hand, the number of SC nuclei expressing Myogenin (Myogenin+) is found to be nearly constant during maturation, while the number of Myogenin+ myonuclei increases significantly. However, fewer SC nuclei and myonuclei express Myogenin in adult birds. These findings suggest a

different role for each of MyoD and Myogenin within active SC nuclei during postnatal development.

4.2 Materials and Methods

4.2.1 Experimental Model

White Leghorn chickens (*Gallus gallus*; Hy-Line W-36, Clark Hy-Line, Brandon, Manitoba) were hatched at the same time and raised under identical conditions at the University of Saskatchewan, Department of Animal and Poultry Science. The birds were put on a 23 hours light, one-hour dark cycle at an initial environment temperature of 35°C. Subsequently, temperature was decreased 3.5°C at weekly intervals until 21°C was reached when the birds were four weeks old. Feeding was done *ad libitum* and initially consisted of commercial chick starter that was replaced with a commercial chick grower (Federated Co-operatives Ltd., Saskatchewan, Canada) when the birds were 42 days old. Chickens usually reach their sexual maturity around the age of 150 days (Limburg 1975). Following the Canadian Council on Animal Care Guidelines, and with the approval of the University of Saskatchewan Committee on Animal Care and Supply, three birds were killed by cervical dislocation at each of the following ages; 9, 30, 62, 79, 117 and 430 days post-hatch. The chicken pectoralis muscle was the model utilized in this study. Some special characteristics of chicken pectoralis are that its superficial regions are composed entirely of type IIb fibers (Rosser et al. 1996), and that myonuclei can be normally located within the central regions the fibers (George and Berger 1966).

This location made it easier to quantify myonuclei because they overly the shadows of the fibers, distinguishing them from interstitial non-muscle nuclei.

4.2.2 Antibodies and Nuclear Labeling Agent

The primary antibodies used for this study were anti-Pax7, anti-MyoD and anti-Myogenin. Anti-Pax7 (Developmental Studies Hybridoma Bank, Iowa, USA) is a mouse monoclonal antibody developed against chicken Pax7 (Berggren et al. 2001). It was used at a dilution of 1:100 with immunocytochemical methods to detect SC nuclei. Anti-MyoD (sc-760, Santa Cruz Biotechnology Inc., California, USA) is a rabbit polyclonal antibody raised against a peptide corresponding to amino acids 1-318 representing full-length MyoD protein of mouse origin. This antibody has been used to detect MyoD in cultured chicken embryo myoblasts (Jiang et al. 1998). In this study, anti-MyoD was utilized at a dilution of 1:500 to detect MyoD+ nuclei by immunocytochemistry and at a dilution of 1:1000 to detect MyoD protein by Western analysis. Anti-Myogenin (sc-576, Santa Cruz Biotechnology Inc., California, USA) is also a rabbit polyclonal antibody raised against amino acids 1-225 representing full-length Myogenin protein of rat origin. It was used at a dilution of 1:50 to detect Myogenin+ nuclei by immunocytochemistry and at a dilution of 1:1000 to detect Myogenin protein by Western blot analysis.

Fluorescein isothiocyanate-conjugated anti-mouse (A-11010) and tetramethyl rhodamine anti-rabbit (A-11001) secondary antibodies (Molecular Probes, Oregon, USA) were used to detect anti-Pax7 (green) and either anti-MyoD or anti-Myogenin

(red) respectively when viewed with epifluorescent microscopy. Each of these secondary antibodies was prepared at a dilution of 1:200. Also, Hoechst 33258 (Bisbenzimide, 10 mg/ml stock concentration, Sigma Chemical Co., Missouri, USA) was applied at a dilution of 1:1,500,000 to label the DNA in nuclei (blue) under epifluorescent microscopy. Horseradish peroxidase secondary antibody (170-6515, Bio-Rad Laboratories, California, USA) was used in Western blot analyses at a dilution of 1:10,000 to visualize labeling by the primary antibodies.

4.2.3 Comparison of Amino Acid Sequencing

The reactivity of anti-MyoD and anti-Myogenin antibodies with chicken MyoD and Myogenin, respectively, was initially determined by comparing the amino acid sequence between these chicken proteins and those from the subjects which these antibodies are known to be reactive against. The comparison was done by using the software programs DNA Strider 1.2 (available for free download on the internet by National Center for Biotechnology Information at http://www.ncbi.nlm.nih.gov, Marck 1988) and Clustal V (available for free download on the internet by National Centre for Biotechnology Information at http://www.ncbi.nlm.nih.gov, Higgins et al. 1992). Comparing the amino acid sequence of chicken MyoD (Lin et al. 1989; Yamamoto and Kuroiwa 2003) with that of human MyoD (Lingbeck et al. 2005; Berkes and Tapscott 2005) or mouse MyoD (Ishibashi et al. 2005; Cao et al. 2006), there was about 86% similarity when conserved amino acid substitutions were considered (Fig. 1). Also, comparing the amino acid sequence of chicken Myogenin (Fujisawa-Sehara et al. 1990) with that of rat Myogenin (Wright et al. 1989) or mouse

Myogenin (Fujisawa-Sehara et al. 1990; Ohkawa et al. 2006), there was about 88% similarity when conserved amino acid substitutions were considered (Fig. 2). These findings indicate that there is a good possibility for these antibodies to react with their corresponding chicken proteins (Dayhoff et al. 1983).

4.2.4 Western Blot Analysis

The specificity of anti-MyoD and anti-Myogenin antibodies against their corresponding chicken proteins was demonstrated by Western blot analyses. The protein extraction and Western protocol were performed similar to Davies et al. (2005) with minor modifications. Protein samples were isolated from age 9 and 49 days post-hatch chicken pectoralis. Also, L8 cultured rat myoblasts were used as a positive control (obtained from American Type Culture Collection, Virginia, USA). L8 myoblast cell line was originally established in 1968 by isolation from primary rat skeletal-muscle cultures prepared from newborn Wistar rats (Yaffe 1968).

4.2.4.1 Tissue Protein Extraction

Chicken pectoralis muscle samples from ages 9 and 49 days post-hatch were obtained and frozen at -80 °C. Each sample, weighing approximately 100 mg, was transferred to a mortar placed over dry ice and crushed with a pestle into powder. After that, the powder sample was placed in 0.5 ml ice-cold Western lysis buffer (20mM HEPES, pH 7.4, 50mM KCl, 0.5mM EDTA, 0.1mM EGTA, 10% glycerol, 1mM DTT, 1X Sigma anti-protease cocktail). A different lysis buffer (Appendix B, point 1) was used in another Western blot experiment that was also applied to detect

the specificity of the anti-MyoD antibody (Appendix D). The sample was then homogenized on ice using a Polytron homogenizer (Brinkmann Instruments, Ontario, Canada) for about 10 min and then sonicated over ice for 15 seconds before being centrifuged for 10 min at 12,128 RCF_{max}. The supernatant was removed and placed in a fresh microtube and Bradford protein determinations were performed (Bradford 1976).

L8 cultured rat myoblasts extract was used as a positive control for Western analyses. L8 cells were collected by centrifugation with 758 RCF_{max} at 4 °C and the pelleted cells resuspended in ice-cold Western lysis buffer (20mM HEPES, pH 7.4, 50mM KCl, 0.5mM EDTA, 0.1mM EGTA, 10% glycerol, 1mM DTT, 1X Sigma anti-protease cocktail). The cells were lysed using multiple freeze-thaw cycles followed by pulse sonication and high-speed centrifugation to remove cell debris (Davies et al. 2005).

4.2.4.2 Gel Electrophoresis and Transblotting

Samples with similar protein concentration were prepared based upon Bradford protein assay using Bio-Rad protein reagent (Bio-Rad Laboratories) and bovine serum albumin (Sigma Chemical Co.) as a standard. Equal amounts of protein (10 μg) from each sample were loaded on an 8% mini-gel, as was 10 μg of a broad range prestained protein molecular weight marker (Fermentas Life Sciences, Ontario, Canada). The samples were then separated by SDS-PAGE performed with 120 Volt at room temperature. Following electrophoresis, the gel was removed and

equilibrated in a fresh transblotting buffer solution for 30 minutes at 4 °C. The proteins were then transblotted from the gel onto a 0.45 μm nitrocellulose membrane (Pall Corporation, Missouri, USA).

4.2.4.3 Western Blot Protocol

Each membrane was blocked with 5% molecular grade fat free skim milk powder (Bio-Rad Laboratories, Ontario, Canada) in phosphate-buffered saline (PBS; 0.02 M sodium phosphate buffer, 0.15 M sodium chloride, pH 7.2) containing 0.1% Tween-20 (Sigma Chemical Co.) for 2 hours at room temperature. The membrane was incubated overnight at 4 °C with either MyoD or Myogenin primary antibodies diluted 1:1000 in 5% skim milk. The membrane was then washed twice with PBS containing 0.1% Tween-20 for 5 min each followed by 3 times with PBS for 5 min each, and then incubated for 30 minutes at room temperature with secondary antibody diluted 1:10,000 in 5% skim milk. An enhanced chemiluminescence detection system (Dupont-NEN, Massachusetts, USA) was used to detect the antigen antibody complexes.

Figure 1: Comparison of amino acid sequence of chicken MyoD to that of human and mouse MyoD. There is about 86% similarity in amino acid sequence between the chicken MyoD protein (298 a.a., 33 kDa) and human or mouse MyoD protein (320 a.a., 34 kDa).

```
chicken-myog    MELFETNPYFFPEQRFYDGENFLGSRLQGYEAAAFPERPEVTLCPESRGA
rat-----myog    MELYETSPYFYQEPHFYDGENYLPVHLQGFEPPGY-ERTELSLSPEARGP
mouse---myog    MELYETSPYFYQEPHFYDGENYLPVHLQGFEPPGY-ERTELSLSPEARGP
                ***.**.***. * .******.*  .***.*.... ** *..* **.**.

chicken-myog    LEEKDSTLPEHCPGQCLPWACKICKRKTVSIDRRRAATLREKRRLKKVNE
rat-----myog    LEEKGLGTPEHCPGQCLPWACKVCKRKSVSVDRRRAATLREKRRLKKVNE
mouse---myog    LEEKGLGTPEHCPGQCLPWACKVCKRKSVSVDRRRAATLREKRRLKKVNE
                ****.   **************.****.**.******************

chicken-myog    AFEALKRSTLLNPNQRLPKVEILRSAIQYIERLQSLLSSLNQQEREQREL
rat-----myog    AFEALKRSTLLNPNQRLPKVEILRSAIQYIERLQALLSSLNQEER---DL
mouse---myog    AFEALKRSTLLNPNQRLPKVEILRSAIQYIERLQALLSSLNQEER---DL
                **********************************.*******.**   .*

chicken-myog    RYRPAA-PQPAAPSECGSGSSSCSPEWSTQLEFGTNPADHLLSDDQAEDR
rat-----myog    RYRGGGGPQPVVPSECNSHSASCSPEWGNALEFGPNPGDHLLAADPTGAH
mouse---myog    RYRGGGGPQPMVPSECNSHSASCSPEWGNALEFGPNPGDHLLAADPTDAH
                *** .. ***  **** * *.******.  **** **.****. * .  .

chicken-myog    NLHSLSSIVESIAVEDVAVTFPEERVQN
rat-----myog    NLHSLTSIVDSITVEDMSVTFPDETMPN
mouse---myog    NLHSLTSIVDSITVEDMSVAFPDETMPN
                *****.***.**.***..*.**.* . *
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Figure 2: Comparison of amino acid sequence of chicken Myogenin to that of rat and mouse Myogenin. There is about 88% similarity in amino acid sequence between the chicken Myogenin protein (227 a.a., 25.8 kDa) and rat or mouse Myogenin protein (224 a.a., 25.2 kDa).

4.2.5 Immunocytochemistry

4.2.5.1 Tissue Preparation and Sectioning

Three muscle samples were excised from the superficial region of the left pectoralis muscle of each bird. Each sample was approximately 0.5 x 0.5 x 1-2 cm. The long axis of each sample was parallel to the direction of the muscle fibers. Each sample was then coated with O.C.T. compound (Miles Inc., Indiana, USA) and immediately frozen in 2-methylbutane cooled via liquid nitrogen (Sewry and Dubowitz 2001). After that, samples were stored at -80°C.

Serial cross-sections of 10 μm thickness were cut using a cryostat at -20°C, and two serial sections were picked up on each ProbeOn Plus microscopic slide (Fisher Scientific Ltd., Ontario, Canada). The reason for collecting two sections on each slide was to increase the possibility of choosing better fields for imaging. Serial slides bearing sections were numbered and stored at -20°C.

4.2.5.2 Immunocytochemistry Protocol

Slides to be treated with anti-MyoD were removed from the freezer and air dried for 15 minutes. Sections were then treated for 30 minutes with 200 μl of blocking solution, which consisted of 5mM EDTA in PBS, 5% goat serum and 1% bovine serum albumin. Blocking solution was drained from each slide onto a paper towel. After that, a cocktail of anti-Pax7 and anti-MyoD primary antibodies diluted in blocking solution was applied over the sections on slides using 150 μl per slide. The slides were kept overnight in the dark at 4°C.

Slides to be treated with anti-Myogenin were removed from the freezer and air dried for 15 minutes. After that, the slides were treated for 10 minutes with 60% acetone in Coplin jars (Fisher Scientific Ltd.). No blocking was used, instead a cocktail of anti-Pax7 and anti-Myogenin primary antibodies diluted in the blocking solution was applied directly over the sections on slides using 150 μl per slide. The slides were kept overnight in the dark at 4°C.

Anti-MyoD or anti-Myogenin slides were then washed three times in fresh PBS solution for five minutes per wash. A cocktail containing the secondary antibodies A-11010 and A11001 was then applied over the sections on each slide for 45 minutes at room temperature, and followed by two five minutes washes in fresh PBS solution. Hoechst 33258 diluted in PBS was then applied over the sections for five minutes, followed by two additional five minutes washes in PBS. All slides were then fixed with 4% formaldehyde in PBS solution for three minutes, and subsequently washed twice for five minutes each in fresh PBS solution. Finally, wet slides were mounted with cover slips using Geltol mounting medium (Thermo Shandon, Pennsylvania, USA) before being examined by fluorescent microscopy.

4.2.5.4 Image Analysis and Data Collection

Five to six different fields of view were captured from slides representing the same animal by using a Zeiss Axioskop 20 microscope equipped for epifluorescence (Carl Zeiss, Oberkochen, Germany) and a Sony (S70) digital still camera (Sony

Electronics Inc., Tokyo, Japan). Three epifluorescent images, each viewed through a different wavelength filter, were acquired from each field. The resultant images were all nuclei in blue, SC nuclei in green and either MyoD+ or Myogenin+ nuclei in red (Fig. 3 and 4). Images were subsequently transferred to a Macintosh G4 computer (Apple Computer Inc., California, USA), and the three images for each field of view were superimposed using Adobe Photoshop (Adobe System Inc., California, USA). The ratios of MyoD+ and Myogenin+ SC nuclei and myonuclei were calculated for at least 100 SC nuclei and 200 myonuclei from each bird. Any Hoechst labeled nucleus that did not superimpose the sillohoutte of the fiber was not calculated as a myonucleus.

4.3 Statistics

After confirming the homogeneity of variance, data were evaluated by one-way analysis of variance (ANOVA). The samples were blocked into six different age groups and the ANOVA was applied at 5% level of significance. When a significant difference was found ($P < 0.05$), Scheffe's post-hoc analysis test was used for a pair wise comparison between different age groups. Also, independent T-test was applied to determine the significance of declination in MyoD and Myogenin expression from maturing (117 days post-hatch) to adult (430 days post-hatch) chickens. All statistical tests were performed using SPSS program (standard version 12.0.0, SPSS Inc.).

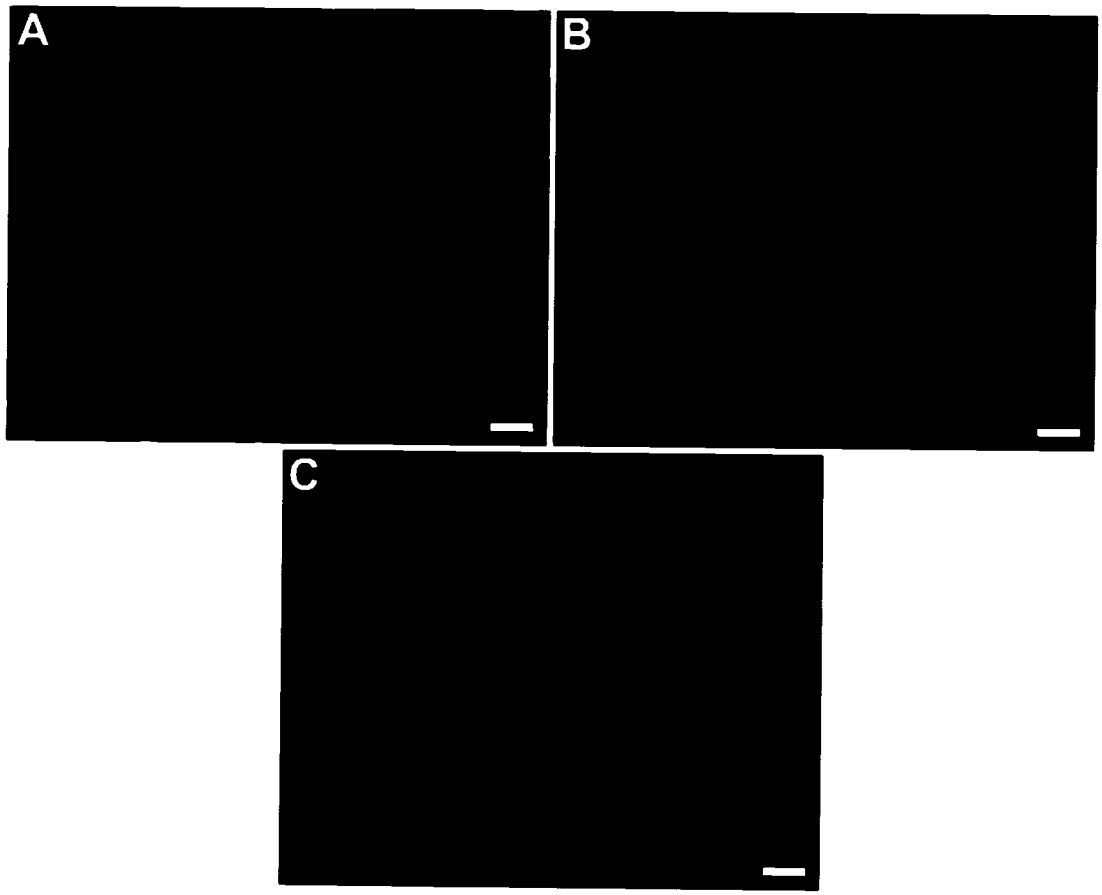

Figure 3: Immunofluorescence labeling of a transverse section of a 62 days old chicken pectoralis to detect MyoD+ nuclei. **(A)** All nuclei appear in blue due to labeling by Hoechst. **(B)** Satellite cell nuclei appear green after labeling by anti-Pax7. **(C)** MyoD+ nuclei appear red after labeling by anti-MyoD. Scale bars = 20 microns.

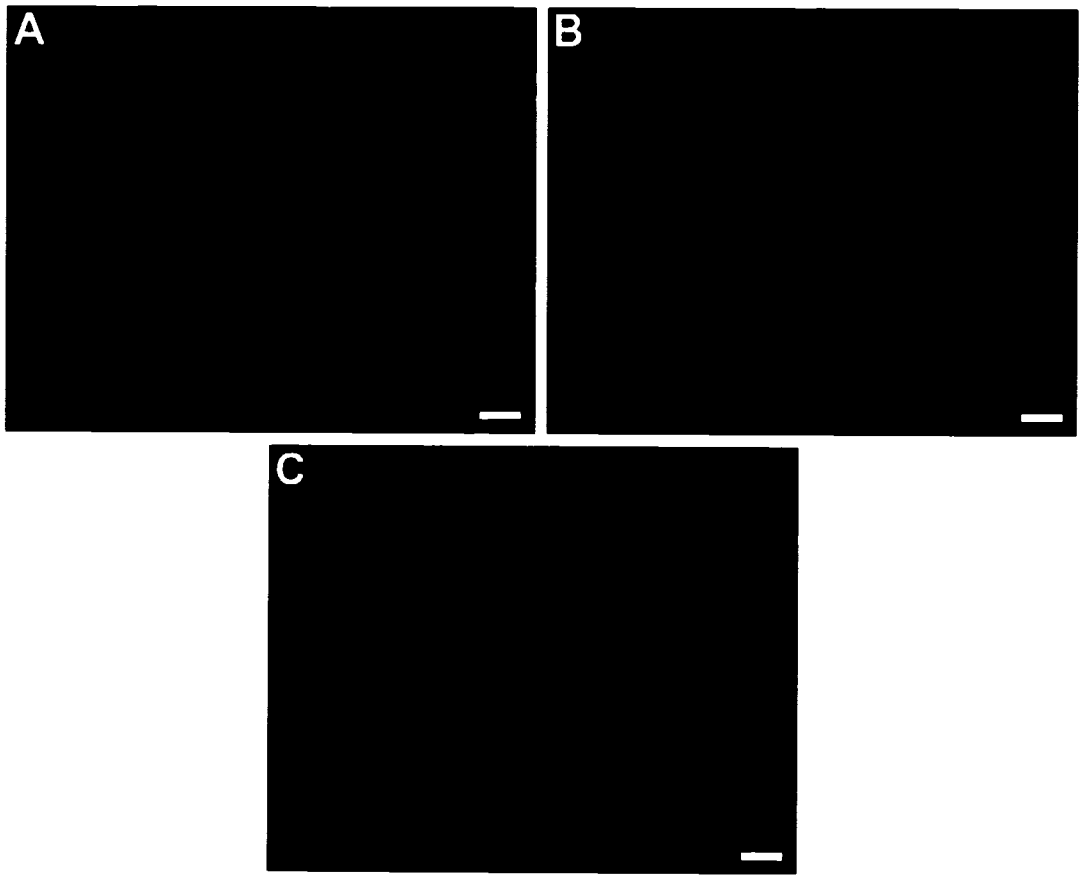

Figure 4: Immunofluorescence labeling of a transverse section of a 62 days old chicken pectoralis to detect Myogenin+ nuclei. **(A)** All nuclei appear in blue due to labeling by Hoechst. **(B)** Satellite cell nuclei appear green after labeling by anti-Pax7. **(C)** Myogenin+ nuclei appear red after labeling by anti-Myogenin. Scale bars = 20 microns.

4.4 Results

4.4.1 Specificity of MyoD and Myogenin Antibodies

The specificity of anti-MyoD and anti-Myogenin antibodies was confirmed by Western blot analyses. Anti-MyoD immunoreactivity was shown as a single band at around 38 kDa (Fig. 5A; Yamamoto and Kuroiwa 2003), while anti-Myogenin immunoreactivity was detected as a single band that appeared at about 36 kDa (Fig. 5B; Fujisawa-Sehara et al. 1990). Also, the immunoreactivity of these antibodies with L8 cell extract confirmed the specificity of these antibodies. These findings indicate that the anti-MyoD and anti-Myogenin polyclonal antibodies used in this study recognize chicken proteins consistent with that predicted for MyoD and Myogenin respectively.

4.4.2 Expression Pattern of MyoD

As illustrated in Figure 6, the percentage of MyoD+ SC nuclei increased significantly ($P < 0.001$) with age. While the percentage of SCs expressing MyoD was 16.67% at age 9 days post-hatch, this value increased to reach 91.67% around maturation at age 117 days. However, this percentage then declined significantly ($P < 0.001$) to 75.69% at 430 days of age.

In myonuclei, the MyoD+ ratio also increased significantly ($P < 0.001$) during maturation. The percentage of MyoD+ myonuclei was 78.50% at age 9 days post-hatch and increased up to 95.17% at 117 days post-hatch. Similar to MyoD labeling

of SCs, this value declined significantly ($P < 0.005$) after maturation to reach 72.50% at age 430 days.

4.4.3 Expression Pattern of Myogenin

The percentage of Myogenin+ SCs increased significantly ($P < 0.01$) at early stages of development from 36.04% at age 9 days to 44.86% at age 30 days (Fig. 7). After that, the proportion was almost constant until maturation and never rose above 47.67%. On the other hand, the percentage of Myogenin+ myonuclei increased significantly ($P < 0.001$) during development from 44.85% at age 9 days post-hatch to 75.28% at 117 days post-hatch. After maturation, the percentages of both Myogenin+ SC nuclei and myonuclei declined significantly to 25.06% ($P < 0.005$) and 20.83% ($P < 0.001$), respectively, at 430 days post-hatch (Fig. 7).

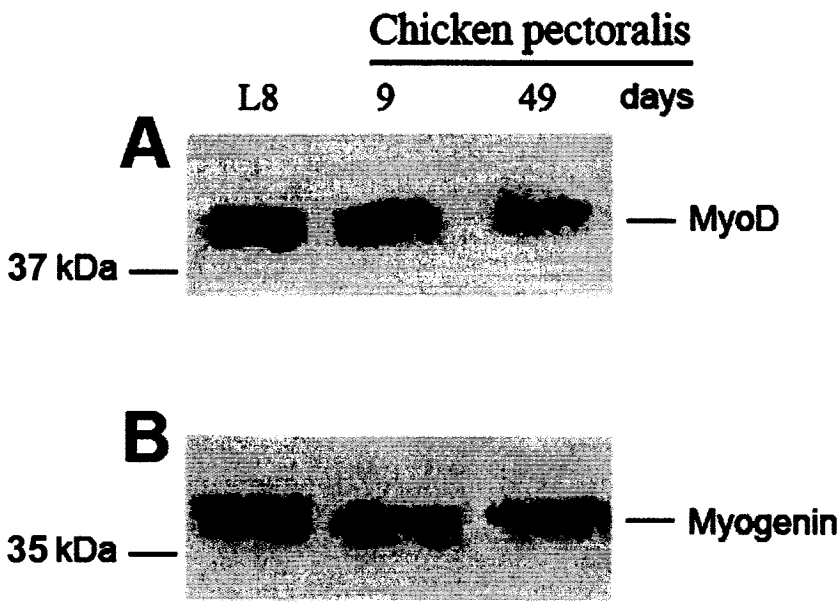

Figure 5: Western blot analyses for anti-MyoD and anti-Myogenin antibodies. **(A)** A Western blot probed with anti-MyoD antibody against chicken pectoralis extracts from age 9 and 49 days post-hatch and L8 cultured rat myoblast extract used as a positive control. **(B)** A Western blot probed with anti-Myogenin antibody against the same samples used in (A). In both analyses, there was only a single band detected on the membrane, suggesting the specificity of these antibodies for their corresponding proteins. See appendices D and E for whole membrane photos of, respectively, MyoD and Myogenin Western blot analyses.

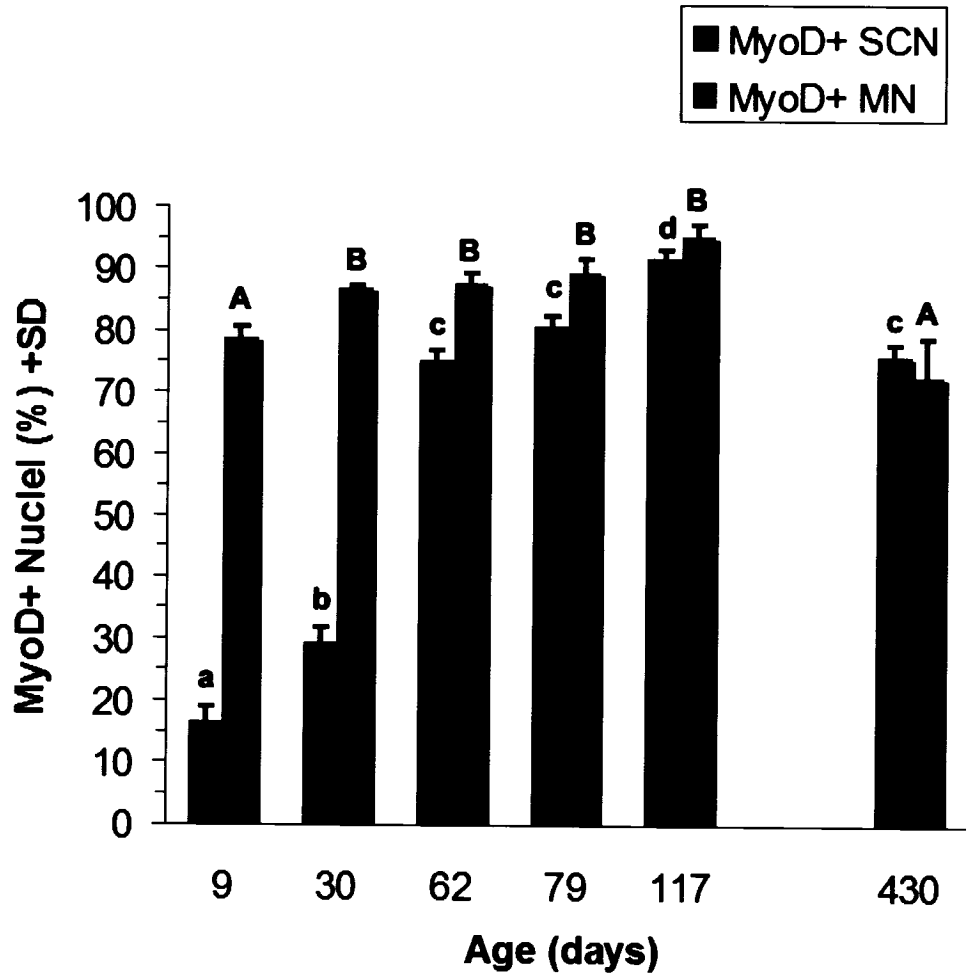

Figure 6: Expression of MyoD protein within satellite cell nuclei (SCN) and myonuclei (MN). The percentages of both MyoD+ satellite cell nuclei and MyoD+ myonuclei increase significantly ($P < 0.001$) during development and reach peak values near maturation at age 117 days post-hatch, where more than 90% of these nuclei are MyoD+. However, this percentage declines after the birds are mature. Columns with different letters are significantly different (Scheffe's, $P < 0.05$). Small letters are used for SCN (green columns) and large letters are used for MN (blue columns).

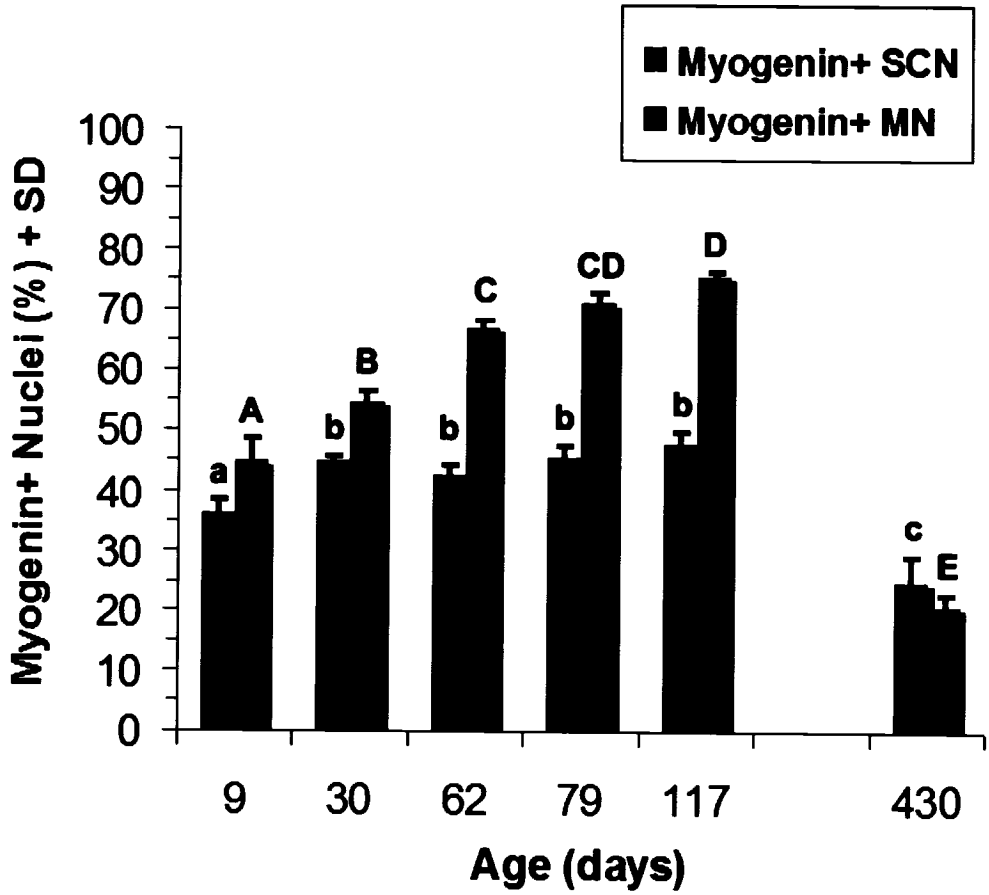

Figure 7: Expression of Myogenin protein within satellite cell nuclei (SCN) and myonuclei (MN). The percentage of Myogenin+ satellite cell nuclei increases significantly ($P < 0.05$) from age 9 days to age 30 days post-hatch. After that, this percentage appears to remain steady during the post-hatch developmental period. It then declines as the birds mature. In myonuclei, Myogenin expression increases significantly ($P < 0.001$) with development until maturation after which it declines. Columns with different letters are significantly different (Scheffe's, $P < 0.05$). Small letters are used for SCN (green columns) and large letters are used for MN (blue columns).

4.5 Discussion

This study uses immunocytochemical techniques to analyze the expression patterns of MyoD and Myogenin during post-hatch development of the chicken. Antibodies developed against MyoD and Myogenin proteins are used to detect MyoD+ and Myogenin+ nuclei, after the specificity of these antibodies is demonstrated by Western analyses. The percentages of MyoD+ and Myogenin+ myonuclei are significantly increased during maturation. On the other hand, while the percentage of MyoD+ SC nuclei increases significantly with maturation, the percentage of Myogenin+ SC nuclei has increased only at early stages of development and then remains nearly constant. At all ages more myonuclei express MyoD than Myogenin. In SCs, at early ages of development, fewer SCs express MyoD than Myogenin. However, as the number of SCs expressing MyoD increases significantly with age, it surpasses the number of SCs that express Myogenin. This difference in expression between MyoD and Myogenin within SC nuclei suggests they may be required at different activated stages of SCs and perhaps play different roles during SC activation.

Previous *in vitro* studies established that the expression of MyoD in cultured myoblasts inhibits their proliferation and enhances myotube formation (Sorrentino et al. 1990; Crescenzi et al. 1990; Montarras et al. 1991; Wilson et al. 2003). On the other hand, lack of MyoD expression in cultured myoblasts enhances proliferation, and impairs their capacity to differentiate (Montarras et al. 1989; Sabourin et al. 1999; Cornelison et al. 2000; Schuierer et al. 2005). Other *in vitro* studies have

shown that MyoD expression could induce terminal cell cycle arrest during myoblast differentiation by increasing the expression of cyclin dependant kinase inhibitor protein p21 (Halevy et al. 1995; Guo et al. 1995). Collectively, these previous works demonstrate that MyoD is required for cessation of myoblast proliferation and transition to differentiation. Myogenin expression was found to always accompany the terminal differentiation stage of cultured myoblasts (Emerson 1990; Montarras et al. 1990; Smith et al. 1994; Yablonka-Reuveni and Paterson 2001). This suggests that Myogenin expression can demarcate the end stage of differentiation within cultured myoblasts and transition towards myotube formation.

These previous *in vitro* studies lead to the conclusion that each of these factors, along with Pax7, can demarcate a specific stage of development within SCs until their differentiation to myonuclei (Fig. 7). The expression of Pax7 can specify SCs in all their different stages; quiescent and active (Seale et al. 2000; Olguin and Olwin 2004; Halevy et al. 2004; Relaix et al. 2005; Kuang et al. 2006). MyoD starts to be expressed at the end stage of SC (Pax7+/MyoD+) proliferation to enhance differentiation and continues after their maturation to myonuclei (Pax7-/MyoD+). The expression of Myogenin demarcates the end stage of differentiation (Pax7+/Myogenin+) and continues after myotube formation (Pax7-/Myogenin+). This is comparable to what Zammit et al. (2006a) have suggested.

The increased number of MyoD+ SCs detected in this study during maturation means that more of these active cells withdraw from the cell cycle and

either move toward differentiation and fusion to muscle fibers, or return to quiescence as the birds grow. The number of Myogenin+ SCs increased significantly (to about 45%) until age 30 days where it remained until maturation. This leads to the assumption that the rate of differentiation increases significantly at very early stages of postnatal development and then remains constant during maturation. These data combined indicate that, as increasing numbers of SCs stop proliferation (MyoD+) during post-hatch growth, a constant percentage of these cells will differentiate to myonuclei (Myogenin+). Presumably, the remaining SCs will move toward G_0 phase to become quiescent later on and lose the expression of MyoD. After maturation, both rates of proliferation and differentiation decline as SCs become more quiescent.

Until now, few *in vivo* studies have investigated the expression and functions of MyoD and Myogenin within skeletal muscle. It has been demonstrated in studies of rodent muscles that MyoD and Myogenin are expressed in active SC nuclei and recently formed myonuclei (Grounds et al. 1992; Fuchtbauer and Westphal 1992; Ishido et al. 2004b). Also, Ishido et al. (2004a) have reported the expression of MyoD and Myogenin within nuclei located in the interstitial spaces between muscle fibers. This study is the first to report the patterns of MyoD and Myogenin expression throughout postnatal development until maturation. Numerous unanswered questions remain, however, regarding the exact functions of MyoD and Myogenin within skeletal muscle during postnatal development. Do these factors interact with each other? Do they play dual roles during the activation of SCs? It is clear that MyoD and Myogenin, along with other myogenic regulators, do not

function alone but rather they participate in a regulatory network that involves a variety of positive and negative factors. Further investigations are still required in order to unveil the complete *in vivo* picture regarding MyoD and Myogenin functions during postnatal development.

In summary, this study reveals that the percentages of MyoD+ and Myogenin+ nuclei are relatively high within growing chicken skeletal muscle. The number of SCs that express MyoD increases significantly during post-hatch development, while Myogenin expression is limited to about half of SC population during this period. It is speculated that MyoD is expressed in late proliferating and in differentiated SCs. This implies that MyoD can distinguish active from quiescent SCs. Myogenin is thought to be expressed at the end of the differentiation stage, where it is positively correlated with the rate of SC differentiation within skeletal muscle.

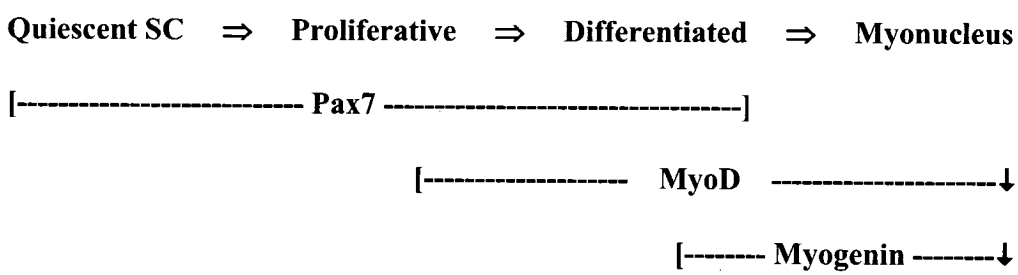

Figure 8: The patterns of expression for Pax7, MyoD and Myogenin factors by satellite cell (SC) nuclei during different developmental stages. Pax7 is expressed by SCs in their quiescent and active states. MyoD expression demarcates the end of proliferation stage and transition towards differentiation. Myogenin expression begins at the terminal stage of SC differentiation.

5.0 CHAPTER

FREQUENCY AND CONCENTRATION OF SATELLITE CELLS IN MEAT PRODUCING CHICKENS AS A NATURAL MODEL OF MUSCLE HYPERTROPHY

5.1 Introduction

Skeletal muscle is composed of long, multinucleated cells known as muscle fibers (Trotter 1991; Allen et al. 1995; Gartner and Hiatt 2001). Postnatal growth of skeletal muscle occurs either through hypertrophy, an increase in fiber size or, to a lesser extent, through hyperplasia, an increase in fiber number (Goldspink 1972; Gollnick et al. 1981; 1983; Alway et al. 1990). These two growth mechanisms depend mainly on the contribution of a specific population of myogenic progenitors termed satellite cells (SCs; Asakura et al. 2002; Mckinnell et al. 2005).

SCs are mononucleated myogenic stem cells situated between the basal lamina and plasmalemma of the muscle fiber and are responsible for postnatal muscle growth and repair (Mauro 1961; Morgan and Partridge 2003; Mckinnell et al. 2005). During growth, active SCs proliferate then differentiate by fusing to a preexisting muscle fiber or by fusing together to form a new fiber (Hawke and Garry 2001; Morgan and Partridge 2003; Jarvinen et al. 2005). Eventually SC nuclei become new myonuclei (Moss and Leblond 1971; Hawke and Garry 2001; Morgan and Partridge 2003). A small portion of SCs, however, do not differentiate but instead revert to quiescence to maintain a self-renewal pool after maturation (Morgan and Partridge 2003; Wozniak et al. 2004).

SCs can be identified by their characteristic position beneath the muscle fiber basal lamina or by their ability to express specific protein markers such as the paired box transcription factor seven (Pax7; Hawke and Garry 2001; Morgan and Partridge 2003; Wozniak et al. 2004). Pax7 is specifically expressed by SCs in all their different stages: quiescent, active, proliferative and differentiated (Seale 2000; Olguin and Olwin 2004; Kuang et al. 2006). An earlier study has confirmed the specific expression of Pax7 by all SCs in the pectoralis muscle of chickens (Halevy et al. 2004).

Previous studies have shown that an increase in myonuclear number is necessary to increase the volume of muscle fibers during postnatal growth (Cheek et al. 1971; Darr and Schultz 1989; Edgerton and Roy 1991). The increased myonuclear number is needed to maintain a constant volume of cytoplasm per myonucleus within the growing fibers (Moss 1968; Allen et al. 1995). The myonuclear domain has been defined as the average volume of cytoplasm associated with a single myonucleus (Rosser et al. 2002). On the other hand, it has been found that actively growing muscle fibers have more SCs (Moss and Leblond 1971; Schultz et al. 1978). For example, the frequency of SCs declines from about 20% in the growing chicken's pectoralis to less than 5% at maturation (Halevy et al. 2004). Since SCs are the main source of new myonuclei, it is surmised that a higher number of SCs is required to meet the greater demand for myonuclei in growing muscle fibers.

This study examines the distribution of SCs and myonuclei in growing meat producing chickens, as a natural model of muscle hypertrophy. This model was chosen because there are some suggestions that experimental models do not represent natural processes (Rosser and George 1987; Mokhtarian et al. 1999). This research provides a chance to compare the frequency and concentration of SCs in the natural model of hypertrophy with that of experimental models studied before. Another important factor for using these chickens is that in the meat production industry very little is known about these birds at the cellular level. Data obtained by this study may be applied to enhance meat production.

The research hypothesis is that there are a greater frequency and concentration of SCs and greater number of myonuclei in hypertrophied muscle of a natural model. These naturally hypertrophied muscles are obtained from the M and Y lines of growing Ross Breeder meat producing chickens, while the control ones are obtained from the control C line. Applying immunocytochemical techniques, including antibody against Pax7 to identify SC nuclei, it was possible to measure the distribution of SCs and myonuclei among these different chicken lines. The frequency and concentration of SCs were significantly ($P < 0.001$) higher in the meat producing lines (M and Y) than in the control (C) line. Also, significantly ($P < 0.001$) greater numbers of SCs and myonuclei per unit length of the fiber were found in the meat producing lines.

5.2 Materials and Methods

5.2.1 Experimental Model

The chicken pectoralis muscle of Ross Breeder meat producing chickens (Ross Breeder Ltd, Newbridge, Scotland) was the experimental model used in this study. The study pool was composed of three distinct lines; the M and Y lines, which are the meat producing birds, and the C-line, from which M and Y lines were selected, acting as the control line. While fast growing M and Y line birds need only six weeks (42 days) to reach market weight, the slow growing C line needs 12 weeks. Following the Canadian Council on Animal Care Guidelines, five birds of each line were sacrificed by cervical dislocation at age 42 days.

5.2.2 Tissue Preparation and Sectioning

The right pectoralis muscle was cut from each bird and weighed. Several muscle samples were excised from the superficial region of the proximal two thirds of each muscle. Each muscle sample was approximately 0.5 X 0.5 X 1.0 – 2.0 cm with its long axis parallel to the direction of the muscle fibers. Each sample was then coated with O.C.T. compound (Miles Inc., Indiana, USA) and immediately frozen in 2-methylbutane cooled via liquid nitrogen (Sewry and Dubowitz 2001). Samples were subsequently stored at -80°C.

Serial cross-sections of 10 μm thickness were cut using a cryostat at -20°C. Two serial sections were picked up on each ProbeOn Plus microscopic slide (Fisher

Scientific Ltd., Ontario, Canada). The reasons for collecting two sections on each slide were to increase the opportunity of choosing better fields for imaging. Serial slides bearing sections were then numbered and stored at -20°C.

5.2.3 Antibodies and Nuclear Labeling Agent

The previously characterized primary antibodies used for immunostaining were anti-Pax7 and anti-laminin. Anti-Pax7 (Developmental Studies Hybridoma Bank, Iowa, USA; Berggren et al. 2001), a mouse monoclonal antibody developed against chicken Pax7, was used at a dilution of 1:100 to detect SC nuclei. Anti-laminin (L9393, Sigma Chemical Co., Missouri, USA; Tisay and Key 1999), a rabbit polyclonal antibody raised against the glycoprotein laminin of mice origin, was used at 1:200 dilution to detect the basal laminae of skeletal muscle fibers.

Fluorescein isothiocyanate-conjugated anti-mouse and tetramethyl rhodamine anti-rabbit secondary antibodies (A-11010 and A-11001, Molecular Probes, Oregon, USA) were used, respectively, to label anti-Pax7 in green and anti-laminin in red when viewed with epifluorescent microscopy. Each of these secondary antibodies was prepared at a dilution of 1:200 in phosphate-buffered saline (PBS; 0.02 M sodium phosphate buffer, 0.15 M sodium chloride, pH 7.2). Hoechst 33258 (Bisbenzimide, 10 mg/ml stock concentration, Sigma Chemical Co., Missouri, USA) was applied at a dilution of 1:1,500,000 in PBS to label the DNA in all nuclei blue under epifluorescent microscopy.

5.2.4 Immunocytochemical Technique

Slides were removed from the freezer and air dried for 15 minutes. Sections were then treated for 30 minutes with 200 µl of blocking solution, consisting of 5mM EDTA in PBS, 5% goat serum and 1% bovine serum albumin. Blocking solution was drained from each slide onto a paper towel. Anti-Pax7 and anti-Laminin primary antibodies diluted in blocking solution were then applied together over the muscle sections on slides using 150 µl per slide. The slides were kept in the dark at 4°C overnight.

After that, slides were washed three times in fresh PBS solution for five minutes per wash. A cocktail containing the secondary antibodies A-11010 and A11001 was then applied over the sections on each slide for 40 minutes at room temperature, and followed by two five minutes washes in fresh PBS solution. Hoechst 33258 was applied over the sections for five minutes, followed by two additional five minutes washes in PBS. All slides were then fixed with 4% formaldehyde in PBS solution for three minutes, and then washed twice for five minutes each in fresh PBS solution. Finally, wet slides were mounted with cover slips using Geltol mounting medium (Thermo Shandon, Pennsylvania, USA) and left to harden for 20 minutes before being examined under epifluorescent microscopy.

5.2.5 Image Analysis and Data Collection

Four to six different fields of view were captured from each immunofluorescent slide using a Zeiss Axioskop 20 microscope equipped for epifluorescence (Carl Zeiss, Oberkochen, Germany) and a Sony (S70) digital still camera (Sony Elctronics, California, USA). Three epifluorescent images were taken for each field with a different wavelength filter. The resultant images were all nuclei in blue, SC nuclei in green and the basal lamina in red (Fig. 1). Images were subsequently transferred to a Macintosh G4 computer (Apple Macintosh, California, USA), and the three images of each field of view were then superimposed using Adobe Photoshop (Adobe System Inc., California, USA).

The ellipse minor axis of 200 contiguous fiber profiles from each animal was measured to assess fiber size. It is defined as the maximum diameter across the lesser aspect of a fiber (Rosser et al. 2000). The basal lamina images were used to measure the ellipse minor axis of individual fibers. The images format was changed to TIFF format, and Scion imaging program (developed by US National Institute of Health; Image J is the current version that is available on the internet at http://rsb.info.nih.gov/ij) was then used to set the images to gray scale to measure the ellipse minor axis.

The numbers of satellite cell nuclei (SCN) and myonuclei (MN) were counted for each of the 200 fibers. After that, the frequency of satellite cells was calculated for each bird using the following formula, frequency = (SCN / SCN+MN) X 100%.

The mean values for SC frequency per group were obtained from five birds in each group.

The number of SCs and number of myonuclei per unit length of fiber were calculated for each bird applying the formula used by Castillo de Maruenda and Franzini-Armstrong (1978). This formula is N= A/(Ln + M), where N is the number of cells per unit length of fiber, A is the mean number of nuclei per fiber profile, Ln is the average length of the nucleus and M is the thickness of tissue section. The mean surface area of the sarcolemma per fiber unit length was also calculated using the formula $S= \pi EU$, where S is the surface area of the plasmalemma per unit length of fiber, E is the ellipse minor axis and U is the unit length of the fiber (1 mm). The area of muscle fiber sarcolemma for each SC was determined by dividing the mean surface area measurement by the mean number of SCs per unit length in each bird. The myonuclear domain size was calculated for each bird by dividing the mean volume of cytoplasm per unit length of fiber by the mean number of myonuclei in that unit length. The volume of cytoplasm per unit length of fiber (V) was computed using the formula $V= \pi(E/2)^2U$. The abbreviations are the same as defined previously.

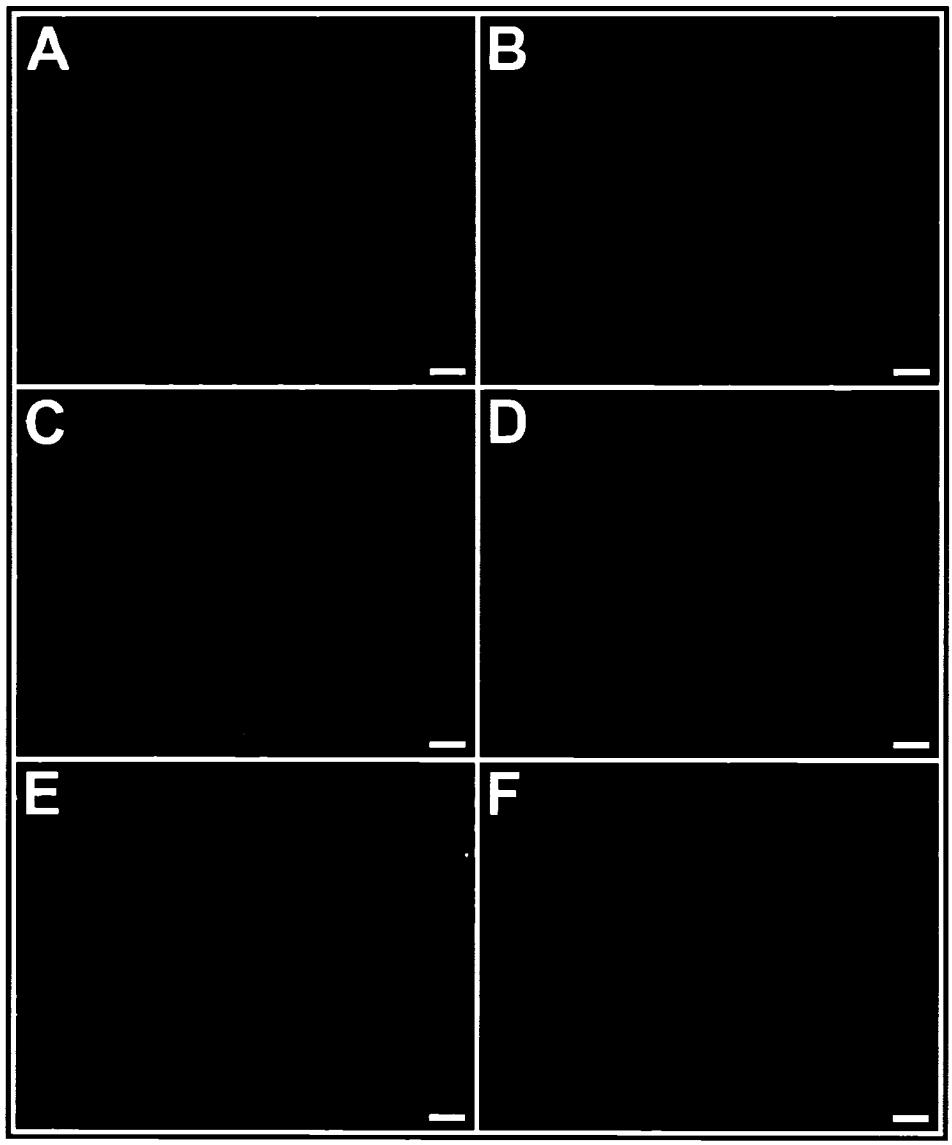

Figure 1: Immunofluorescent identification of satellite cell nuclei in transverse sections from pectoralis muscles of the C, M and Y lines of Ross Breeder chickens at age 42 days post-hatch. **A** and **B** represent a section taken from the pectoralis muscle of a control C line bird, while **C** and **D** are taken from the pectoralis of an M-line bird, and **E** and **F** from the pectoralis muscle of a Y-line bird. **A**, **C** and **E** show all nuclei labeled in blue by Hoechst and basal laminae in red labeled by anti-laminin. **B**, **D** and **F** reveal satellite cell nuclei labeled in green by anti-Pax7 and basal laminae in red labeled by anti-laminin. Scale bars = 30 μm.

5.3 Statistics

After determining the homogeneity of variance, data were evaluated by the one-way analysis of variance (ANOVA) at 5% level of significance. The samples were blocked into three different groups and ANOVA was applied. If a significant difference was found ($P < 0.05$), Scheffe's post-hoc analysis test was applied to examine the statistical differences between groups. All statistical analyses were performed using SPSS program (standard version 12.0.0, SPSS Inc.).

5.4 Results

5.4.1 Size of Fibers' Cross Sectional Profiles

The ellipse minor axis was the measurement used to quantify the size of fibers among the three different bird lines. The mean size of fiber profile was significantly ($P < 0.001$) larger in both the M and Y line birds than in the birds of the C line (Fig. 2). The mean ellipse minor axis ± standard error (SE) in C-line birds was 31.73 ± 1.67 μm, while it was 51.76 ± 1.31 μm in birds of M line and 54.17 ± 1.44 μm in Y line birds.

5.4.2 Frequency of SCs among M, Y and C lines

As evident in Figure 3, the frequency of SCs was significantly ($P < 0.001$) higher in birds of both the M and Y lines compared to those of the control C line. On the other hand, there was no significant ($P = 0.33$) difference in SC frequency between birds of the M and Y lines. The mean frequency in C line birds ± SE was

10.85 ± 0.36 %. This value increased to 14.10 ± 0.32 % in the M line birds, and to 13.57 ± 0.08 % in the Y line birds.

5.4.3 Number of SCs per Unit Length of Fiber

The number of SCs along a mm of fiber was significantly ($P < 0.001$) higher in the M and Y line Birds than in the C line birds. In the control C line birds, the mean number of SCs per mm segment of the fiber ± SE was 11.50 ± 0.32. This number was 27.65 ± 1.38 in M line birds and 24.60 ± 1.03 in Y line birds.

5.4.4 Surface Area of Sarcolemma per SC

The surface area of muscle fiber sarcolemma was calculated for each of the three bird lines. There was significantly ($P < 0.001$) less surface area of sarcolemma per SC in the M and Y lines than in the C line birds (Fig. 4). The mean surface area per SC ± SE in the C line group was 8,667.40 ± 428.99 μm^2. This value was 5,841.37 ± 198.46 in M line group and 6,937.35 ± 189.31 in Y line group. These results indicate that there is a greater concentration of SCs in the M and Y line birds than in the control C line birds.

5.4.5 Number of Myonuclei per Unit Length of Fiber

There was significantly ($P < 0.001$) more myonuclei per mm of fiber in the M and Y lines than in the C line. The mean myonuclear number per mm of fiber ± SE in the C line was 95.00 ± 4.56. This number was 168.00 ± 4.64 in M line and 157.00 ± 6.24 in Y line.

5.4.6 Myonuclear Domain Size

The size of myonuclear domain (volume of cytoplasm per myonucleus) was measured for each line. Surprisingly, the myonuclear domain was significantly ($P < 0.001$) larger in both the M and Y line birds than in those of the C line (Fig. 5). The mean myonuclear domain size in the C line group ± SE was approximately 8,334.17 ± 551.03 μm^3. This mean size value was 12,532.52 ± 417.07 μm^3 in M line group and 14,716.01 ± 575.86 μm^3 in Y line group.

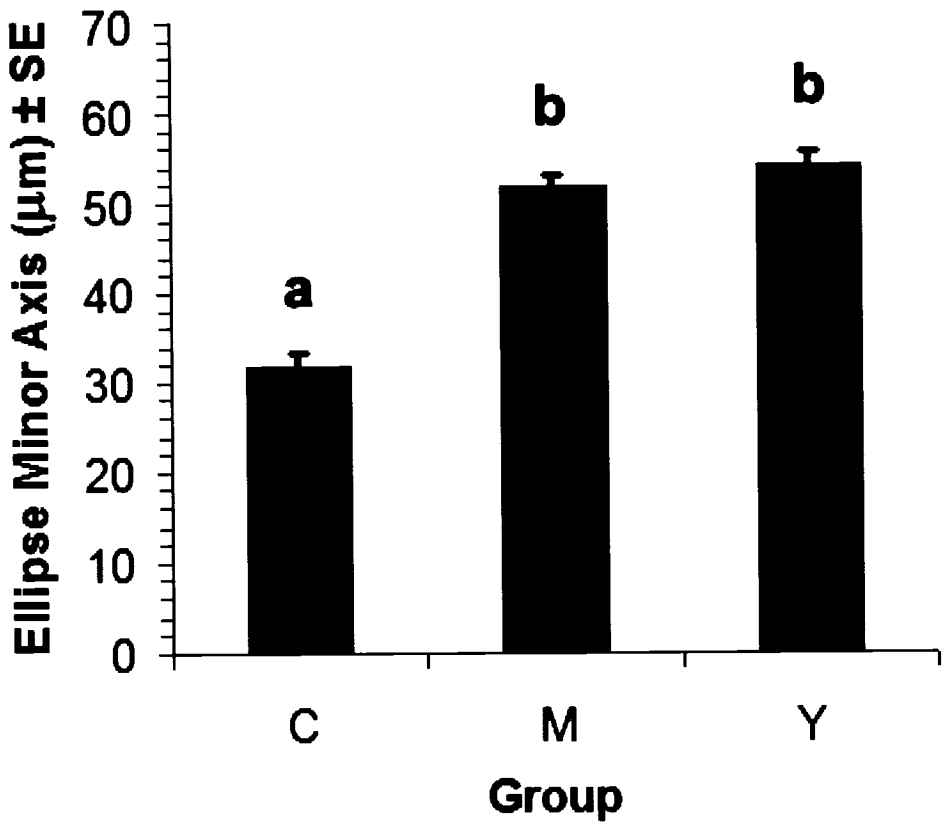

Figure 2: Size of fiber cross sectional profiles in the pectoralis muscle of the C, M and Y lines of Ross Breeder chickens at age 42 days post-hatch. The ellipse minor axis was measured for the pectoralis muscle fibers from five birds in each line. Each column represents the mean ellipse minor axis in micrometers (µm) ± standard error (SE). The minor axis is found to be significantly ($P < 0.001$) larger in the selected meat producing M and Y lines, which represent the naturally hypertrophied model, than in the control C line. Columns with different letters are significantly different (Scheffe's, $P < 0.05$).

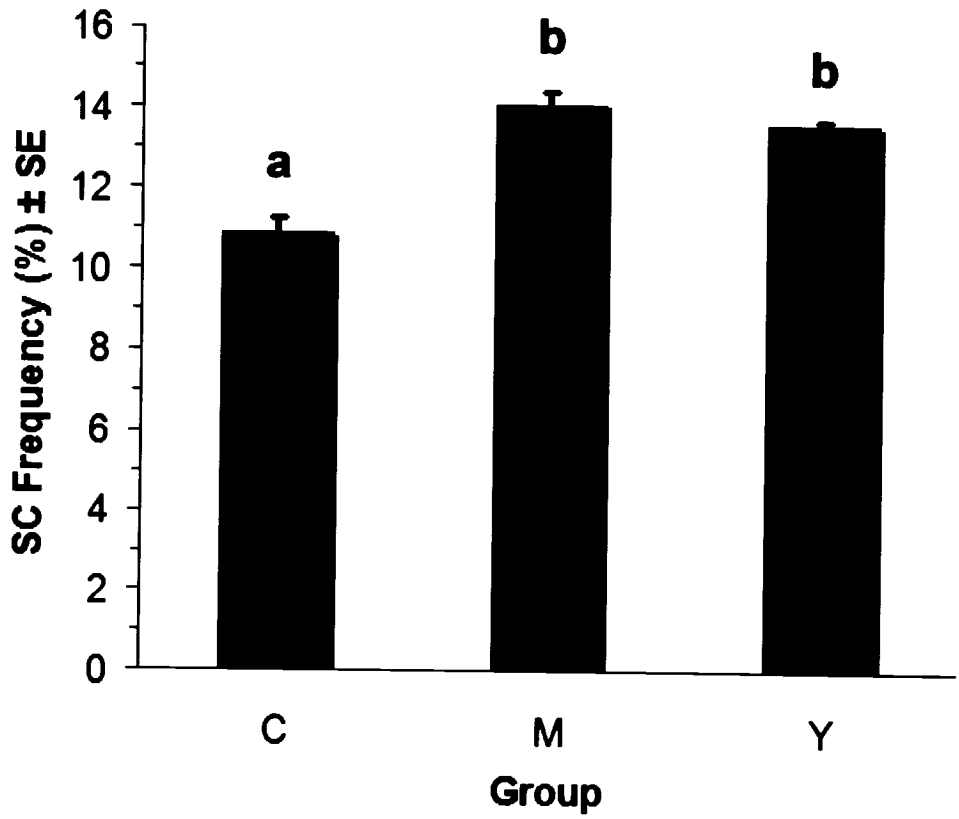

Figure 3: Satellite cell (SC) frequency in the pectoralis muscle of the C, M and Y lines of Ross Breeder chickens at age 42 days post-hatch. The frequency of SCs, (SC nuclei/SC nuclei + myonuclei) x 100%, was obtained from five birds in each line. Numbers of SC nuclei and myonuclei were counted separately in 200 contiguous fibers for each muscle sample. Each column represents mean SC frequency ± standard error (SE). The frequency is significantly ($P < 0.001$) greater in both hypertrophied fibers of the M and Y lines than those in the control C line. Columns with different letters are significantly different (Scheffe's, $P < 0.05$).

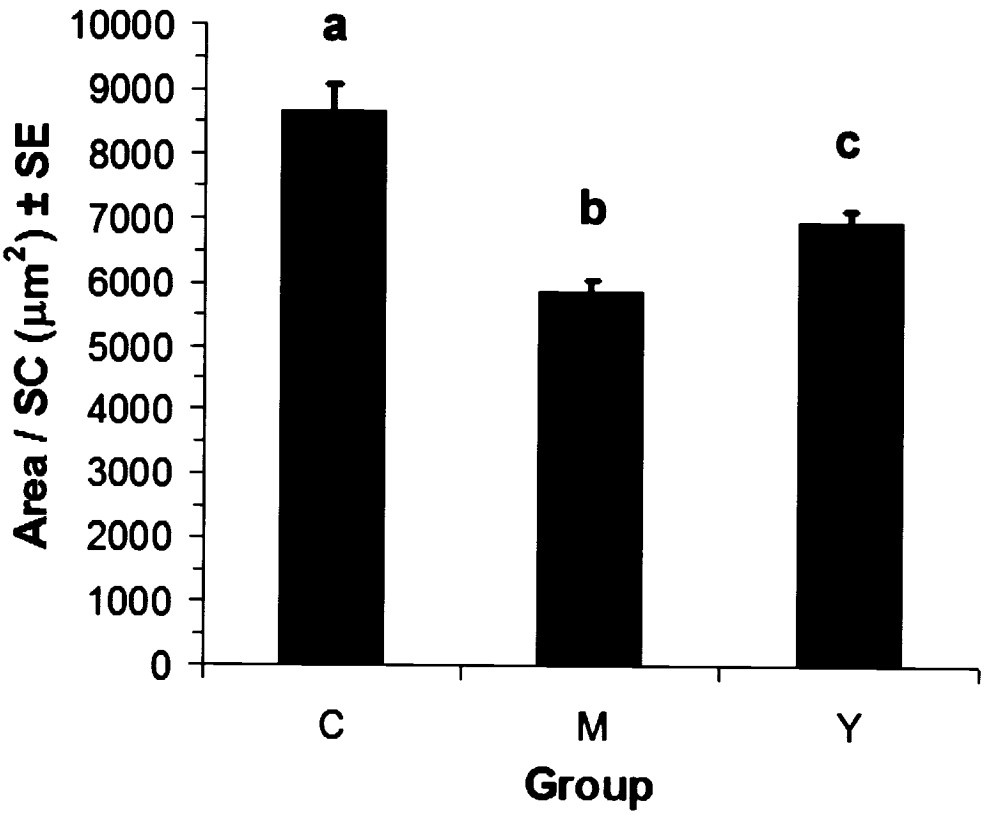

Figure 4: Surface area of sarcolemma per satellite cell (SC) in the pectoralis muscle of the C, M and Y lines of Ross Breeder chickens at age 42 days post-hatch. The area of sarcolemma (μm^2) for each SC was measured in five birds from each line. Each column represents the mean area of sarcolemma per SC ± standard error (SE). There is significantly ($P < 0.001$) less area per SC in the M and Y lines than in the C line. This means that SCs are located closer together in M and Y lines than in the C line birds. Columns with different letters are significantly different (Scheffe's, $P < 0.05$).

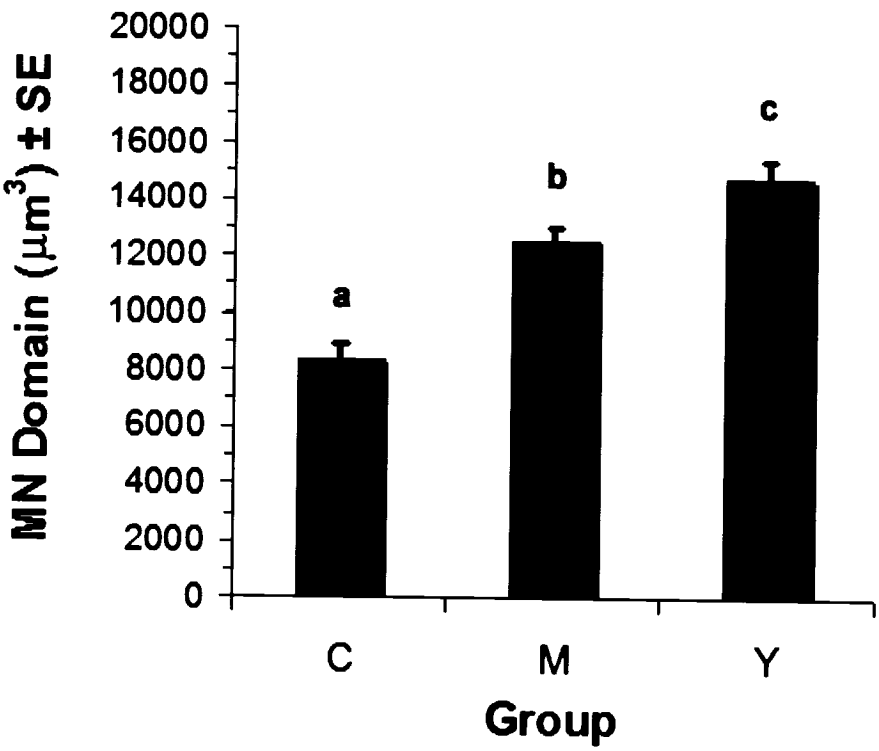

Figure 5: Size of myonuclear domain in pectoralis muscle of the C, M, and Y lines of Ross Breeder chickens at age 42 days post-hatch. The myonuclear domain size was measured in the pectoralis muscle of five birds from each line. Each symbol represents the mean myonuclear domain size (μm^3) ± standard error (SE). The domain size is significantly ($P < 0.001$) larger in M and Y lines than in the C line birds. Columns with different letters are significantly different (Scheffe's, $P < 0.05$).

5.5 Discussion

This study investigates the distribution of SCs and myonuclei in the growing hypertrophied fibers of Ross Breeder's strain of meat producing chickens. These birds offer a natural model of muscle fiber hypertrophy. Various methods have been reported by many researchers to induce muscle fiber hypertrophy in both humans and laboratory animals. A well known and established method is exercise-induced muscle hypertrophy (Goldspink and Howells 1974; Gonyea 1980; Kadi and Thornell 2000). In this method, the hypertrophy is induced by prolonged exercise regimens under normal physiological conditions. Another method known as compensatory hypertrophy, has also been used to enhance muscle growth (Gollnick et al. 1981; Salleo et al. 1983; Timson et al. 1985). Three procedures inducing compensatory hypertrophy have been reported: tenotomy (severing the tendon of the synergistic muscle), ablation (complete or partial removal of synergistic muscle) and denervation (cutting of the synergistic muscle motor neuron). In birds, a common method used to produce hypertrophy in wing muscles is the stretch-induced hypertrophy (Holly et al. 1980; Alway et al. 1989a; Antonio and Gonyea 1993). In this method a weight is hung from the tip of the bird's wing for a sufficient period of time. However, the hypertrophy resulting from the stretch-induced model seems to be associated with inflammation and edema in the muscle and therefore it cannot be compared with physiological muscle hypertrophy. The present study is unique in that it is the first to measure SC distribution in a natural model of enlarged fibers.

This study demonstrates that the hypertrophied fibers of growing muscles have a greater frequency, number and concentration of SCs. Also, it shows a greater number of myonuclei in the hypertrophied fibers than in the normal control ones. Growing fibers require a higher rate of protein synthesis and this is achieved through supplying more myonuclei (Edgerton and Roy 1991; Allen et al. 1999; Ohira et al. 2001). Also, myonuclei arise from SCs (Seale and Rudnicki 2000; Hawke and Garry 2001). Thus, the greater numbers of SCs and myonuclei observed in the growing hypertrophied fibers is presumably correlated with the high demand for protein synthesis in these fibers.

Previous studies have reported a positive correlation between muscle hypertrophy, and the numbers of SCs and myonuclei. Kadi and Thornell (2000) observed an increase in the numbers of SCs and myonuclei within human trapezius muscle after a strength training program. Testosterone-induced muscle hypertrophy was also associated with an increase in the number of SCs and a proportional increase in the number of myonuclei (Sinha-Hikim et al. 2003). Winchester et al. (1991) demonstrated that SCs are induced to enter the cell cycle and proliferate in stretch-enlarged anterior latissimus dorsi muscle of the adult quail. Similar to these earlier studies conducted on experimental models of hypertrophy, the natural model of hypertrophy studied here shows the same association between enlarged muscle fibers and the increased numbers of SCs and myonuclei.

Even though the number of myonuclei per mm of fiber was greater in the M and Y lines, the size of myonuclear domain was much larger in these two lines than in the control line. Although some previous studies suggested that smaller domain size, where myonuclei are closer together, indicates a high rate of protein synthesis and growth (Knizetova et al. 1972; Edgerton and Roy 1991), other studies showed that atrophy of hindlimb muscle fibers was also associated with smaller myonuclear domain size (Allen et al. 1996, 1997; Kasper and Xun 1996a, 1996b; Verheul et al. 2004). Still, it has not been shown that larger domains have a slower rate of growth or less active myonuclei. Chen et al. (2002) mentioned that enhanced transcriptional and translational activities of myonuclei lead to expansion of the myonuclear domain size. Therefore, it can be extrapolated that the size of myonuclear domain cannot always be used as an indicator of growth activity, and that myonuclei in the M and Y line birds can be actively involved in protein synthesis. An explanation for this might be that the rate of fiber growth is much faster than the rate of myonuclei formation from SCs. Kadi and Thornell (2000) reported an increased myonuclear number within hypertrophied fibers of human trapezius muscle, and suggested this increase is to maintain an optimal cytoplasm-to-myonuclear ratio. However, they did not attempt to measure the size of myonuclear domain in their study. In 2005, the same researchers reported a larger myonuclear domain size within hypertrophied fibers of human vastus lateralis muscle (Eriksson et al. 2005).

The difference in growth rate between meat and control birds can be attributed to genetic and nutritional factors (Grossmann et al. 2004). Meat birds were found to exhibit a higher level of growth hormone receptor mRNA transcripts within skeletal muscle tissue compared to control birds (Zhao et al. 1998). Also, significantly higher plasma IGF-1 concentrations were detected in hypertrophied meat producing chickens compared to normal chickens (Zhao et al. 1996). Meat producing chickens usually receive a high protein diet. Feeding normal chickens with this high protein diet resulted in 35% increase of body weight at age 42 days post-hatch, when compared to chickens that received the normal diet (Grossmann et al. 2004).

In conclusion, the results of this study are consistent with that of earlier studies conducted on experimental models of hypertrophy. This suggests that these experimental models are valid and resemble the natural process of hypertrophy. The frequency and concentration of SCs as well as the number of myonuclei are found to be greater in hypertrophied fibers of meat producing chickens compared to normal fibers of the control birds. Concomitantly, there has been a larger myonuclear domain size in the M and Y hypertrophied fibers than in the control C line fibers. This may be an indication of a high myonuclear activity within M and Y line fibers.

6.0 CHAPTER

THE EFFECTS OF NANDROLONE DECANOATE ON THE DISTRIBUTION OF SATELLITE CELLS AND THE MORPHOLOGY OF SKELETAL MUSCLE FIBERS

6.1 Introduction

Vertebrate skeletal muscle fibers are elongated multinucleated cells (Trotter 1991; Allen et al. 1995; Gartner and Hiatt 2001). These fibers are unusual in that they do not undergo division during postnatal life. Instead, growth of skeletal muscle occurs by increase in the size of the fibers or, to a very limited extent, by formation of new fibers (Goldspink 1972; Alway et al. 1990). These two growth mechanisms, known as hypertrophy and hyperplasia respectively, depend mainly on the contribution of a specific population of myogenic precursors known as satellite cells (SCs).

SCs are mononucleated myogenic stem cells that reside between the basal lamina and plasmalemma of the muscle fiber (Mauro 1961; Mckinnell et al. 2005; Zammit et al. 2006a). They contribute to muscle growth and repair by fusing and adding new myonuclei to the fiber (Moss and Leblond 1971; Hawke and Garry 2001). Under normal conditions, SCs are usually quiescent in their characteristic location beneath the basal lamina. When there is a need for growth or repair in the muscle, SCs become active and start to proliferate. After that, the daughter cells fuse to the fiber and differentiate to become myonuclei. In a less common scenario, SCs can fuse together to form new myofibers (Hawke and Garry 2001; Dhawan and Rando 2005; Zammit et al. 2006a). The remaining cells revert to a quiescent state to maintain a reservoir of these cells (Wozniak et al 2005; Dhawan and Rando 2005; Zammit et al. 2006a).

Identification of SCs is made based upon either their characteristic location beneath the basal lamina, or by detecting specific proteins expressed only by SCs such as the paired box transcription factor 7 (Pax7), M-Cadherin or CD 34 (Hawke and Garry 2001; Morgan and Partridge 2003). The advantage of Pax7 is that it is expressed by SCs during all their stages: quiescent, proliferative and differentiated (Seale et al. 2000; Olguin and Olwin 2004; Relaix et al. 2005; Kuang et al. 2006). In the previous study in chapter 3.0 the specific expression of Pax7 by all SCs in the pectoralis muscle of post-hatch chicken was confirmed.

Anabolic androgenic steroids (AAS) are synthetic derivatives of the endogenous primarily male steroid hormone, testosterone. The anabolic effect of AAS helps the body retain dietary protein, thereby aiding growth of muscles, bone and skin. The androgenic properties of AAS include the activation of male reproductive system and development of secondary sexual characteristics, such as hair distribution and musculoskeletal configuration (Ciccero and O'Connor 1990; Mottram and George 2000; Kuhn 2002). AAS were originally developed for treatment of hypogonadal dysfunction, commencement of delayed puberty in men and for growth promotion (Basaria et al. 2001). However, due to their anabolic effects, AAS have become vastly popular among athletes of different kinds of sports. It is already established that AAS increase skeletal muscle mass, strength and endurance (Lukas 1993; Bhasin et al. 1996; Wouter et al. 2004). Yet, the exact mechanisms by which they do so are poorly understood. Sinha-Hikim et al. (2004) have confirmed the localization of androgenic receptors in SC nuclei. Activation of

SCs by AAS is thought to be one of the mechanisms by which these drugs can lead to the hypertrophy of skeletal muscles.

The steroid Nandrolone Decanoate is one of the most popular and commonly used anabolic steroids (Perry et al. 1990; Verroken 2001). Its popularity is likely due to the fact that it exhibits significant anabolic effects with minimal androgenic side effects. This makes Nandrolone a very well tolerated drug that is useful for helping in the treatment of a wide variety of clinical conditions, such as chronic obstructive pulmonary diseases (Creutzberg et al. 2003), osteoporosis and osteopenia (Flicker et al. 1997; Li et al. 2000), joint pain (Leardini et al. 1981; Bird et al. 1987) and also for HIV. In HIV patients, Nandrolone has been shown to be effective at safely increasing the lean bodyweight of these patients (Cuerda et al. 2005; Gold et al. 2006). A recommended intramuscular therapeutic dose of Nandrolone is suggested to be 0.4 mg/kg/day in humans (Tamaki et al. 2003). However, this dose can vary widely based on the type of the medical condition and on its severity.

Nandrolone is also the most widespread anabolic steroid used by athletes and especially bodybuilders for muscle buildup (Wouter et al. 2004). It is also known as the anabolic steroid Deca Durabolin. Its anabolic activity on muscle tissue is thought to be superior to that of testosterone (Shahidi 2001). However, it appears less effective than testosterone in producing the androgenic effects on other tissues such as; skin and nervous tissue. Nandrolone has a half-life in the muscle of approximately six days, and the duration of its effect can last up to three weeks (Van

der vies 1993). Until now there has been no study aimed to detect the direct effects of Nandrolone on the distribution of SCs and myonuclei.

This research examines the effects of Nandrolone Decanoate on the number and distribution of SCs, and on the morphology of skeletal muscle fibers during the development of chicken pectoralis muscle. It tests the hypothesis that Nandrolone administration leads to increased muscle mass and fiber size within the growing chicken pectoralis, and the hypothesis that there is a greater frequency and a greater concentration of SCs in Nandrolone treated birds than in the controls. The birds were divided into two groups. The first group received the Nandrolone injections while the second group received normal saline injections as a control. The numbers of SC nuclei and myonuclei along with the size of muscle fibers were measured by using immunocytochemical techniques. There was a significant ($P < 0.01$) increase in fiber size within Nandrolone treated group. Also, the frequency and concentration of SCs were both significantly ($P < 0.05$) higher in Nandrolone group than in the control one. This study indicates that Nandrolone can induce skeletal muscle fiber hypertrophy and this is correlated with increased numbers of SCs.

6.2 Materials and Methods

6.2.1 Experimental Model

Female White Leghorn chickens (*Gallus gallus*; Hy-Line W-36, Clark Hy-Line) were hatched at the same time and raised under identical conditions at the University of Saskatchewan, College of Veterinary Medicine. The birds were put in a large room and housed on the floor with aspen shaving bedding and were fed *ad libitum*. The birds were exposed to 24 hours light in the first three days and then kept at 12 hours light and 12 hours dark cycle. The initial room temperature was 35°C. Subsequently, the temperature was decreased 2.5°C at weekly intervals until 20°C was reached when the birds were 42 days old. The birds were then marked with two different colors (blue and red) to identify the control group (blue) from the Nandrolone group (red). At age 63 days, the injections into the left pectoralis muscle of the birds were begun. Four injections were given for each group on a weekly basis. The control group received 300 ml normal saline per injection, while the Nandrolone group received 300 ml (30 mg) Nandrolone Decanoate (Organon, Oss, Holland) per injection. Following the Canadian Council on Animal Care Guidelines, and with the approval of the University of Saskatchewan Committee on Animal Care and Supply, four birds were killed per group by cervical dislocation at the age of 91 days post-hatch.

The chicken pectoralis muscle was the model utilized in this study. Chicken pectoralis is a very large muscle, which makes it easier to handle, and it is composed almost entirely of type IIb fibers. In addition to the pectoralis, two leg muscles,

iliotibialis cranialis and gastrocnemius externus (pars lateralis), were excised and weighed from each bird. The reasons for weighing these leg muscles were to ensure that the effect of Nandrolone is systemically distributed and not localized to the region of injection. Since chickens use their legs more constantly, it is expected that the increase in muscle mass would be more prominent in the leg muscles.

6.2.2 Tissue Preparation and Sectioning

Three muscle samples were then excised from the cranial half of the superficial region of the main part of the left pectoralis muscle (M. pectoralis pars sternobrachialis; Vanden Berge and Zweers 1993) of each bird. Each sample was approximately 0.5 x 0.5 x 1.0-2.0 cm. The long axis of each sample was parallel to the direction of the muscle fibers. Each sample was then coated with O.C.T. compound (Miles Inc., Indiana, USA) and immediately frozen in 2-methylbutane cooled via liquid nitrogen (Sewry and Dubowitz 2001). After that, samples were stored at -80°C.

Serial cross-sections of 10 μm thickness were cut at -20°C using a cryostat. Two serial sections were picked up on each ProbeOn Plus microscopic slide (Fisher Scientific Ltd., Ontario, Canada). The reasons for collecting two sections on each slide were to increase the possibility of choosing better fields for imaging. Serial slides bearing sections were numbered and stored at -20°C.

6.2.3 Antibodies and Nuclear labeling Agent

The previously characterized primary antibodies used for immunostaining were anti-Pax7 and anti-laminin. Anti-Pax7 (Developmental Studies Hybridoma Bank, Iowa, USA; Berggren et al. 2001), a mouse monoclonal antibody developed against chicken Pax7, was used at a dilution of 1:100 to detect SC nuclei. Anti-laminin (L9393, Sigma Chemical Co., Missouri, USA; Tisay and Key 1999) a rabbit polyclonal antibody raised against the glycoprotein laminin of mice origin, was used at 1:200 to detect the basal laminae of skeletal muscle fibers.

Fluorescein isothiocyanate-conjugated anti-mouse and tetramethyl rhodamine anti-rabbit secondary antibodies (A-11010 and A-11001, Molecular Probes, Oregon, USA) were used to detect anti-Pax7 in green and anti-laminin in red respectively when viewed with epifluorescent microscopy. Each of these secondary antibodies was prepared at a dilution of 1:200 in phosphate-buffered saline (PBS; 0.02 M sodium phosphate buffer, 0.15 M sodium chloride, pH 7.2). Hoechst 33258 (Bisbenzimide, 10 mg/ml stock concentration, Sigma Chemical Co., Missouri, USA) was applied at a dilution of 1:1,500,000 in PBS to label the DNA in all nuclei blue under epifluorescent microscopy.

6.2.4 Immunocytochemical Protocol

Slides were removed from the freezer and air dried for 15 minutes. Sections were then treated for 30 minutes with 200 µl of blocking solution, which consisted of 5mM EDTA in PBS, 5% goat serum and 1% bovine serum albumin. Blocking solution was drained from each slide onto a paper towel. After that, anti-Pax7 and anti-laminin primary antibodies diluted together in blocking solution were applied over the sections on slides using 150 µl per slide. The slides were kept overnight in the dark at 4°C.

Slides were then washed three times in fresh PBS solution for five minutes per wash. A cocktail containing the secondary antibodies A-11010 and A11001 diluted in PBS was then applied over the sections on each slide for 40 minutes at room temperature, and followed by two five minutes washes in fresh PBS solution. Hoechst 33258 was applied over the sections for five minutes, followed by two additional five minutes washes in PBS. All slides were then fixed with 4% formaldehyde in PBS solution for three minutes, and then washed twice for five minutes each in fresh PBS solution. Finally, wet slides were mounted with cover slips using Geltol mounting medium (Thermo Shandon, Pennsylvania, USA) and left for 20 minutes to harden before being examined under epifluorescent microscopy.

6.2.5 Image Analysis and Data Collection

Five different fields of view were captured from each immunofluorescent slide using a Zeiss Axioskop 20 microscope equipped for epifluorescence and a Sony (S70) digital still camera. Three epifluorescent images, each viewed through a different wavelength filter, were acquired from each field. The resultant images were all nuclei in blue, SC nuclei in green and basal lamina in red (Fig. 1). Images were subsequently transferred to a Macintosh G4 computer, and the three images of each field of view were superimposed using Adobe Photoshop (Adobe System Inc., California, USA).

The ellipse minor axis of 200 contiguous fiber profiles from each animal was measured to assess fiber size. The basal lamina images were used to measure the ellipse minor axis of individual fibers. The images format was changed to TIFF format and Scion imaging program (developed by US National Institute of Health; Image J is the current version that is available on the internet at http://rsb.info.nih.gov/ij) was then used to set the images to gray scale to measure the ellipse minor axis.

The numbers of satellite cell nuclei (SCN) and myonuclei (MN) were counted for each of the 200 fibers. The frequency of satellite cells was then calculated for each animal using the formula: frequency = (SCN / SCN+MN) X 100%. The mean values for SC frequency per group were obtained from four animals in each group.

The number of SCs and myonuclei per unit length of fiber was also calculated for each bird using the formula of Castillo de Maruenda and Franzini-Armstrong (1978). This formula is: N= A/(Ln + M), where N is the number of cells per unit length of fiber, A is the mean number of nuclei per fiber profile, Ln is the average length of the nucleus and M is the thickness of tissue section. The surface area of the sarcolemma per unit length of the fiber was also measured using the formula: S= πEU, where S is the surface area of the sarcolemma per unit length of fiber, E is the ellipse minor axis and U is the unit length of the fiber (1 mm). After that, the area of muscle fiber sarcolemma per each SC was determined by dividing the surface area measurements by the number of SCs in each bird. The myonuclear domain size was calculated for each bird by dividing the volume of cytoplasm per unit length of fiber by the number of myonuclei in that unit length. The volume of cytoplasm per unit length of fiber (V) was computed using the formula $V= \pi(E/2)^2U$.

6.3 Statistics

The samples were blocked into two groups (control Vs. Nandrolone). Levene's test for equality of variances was first applied to determine the homogeneity of variance. Data were then evaluated by independent samples t-test at 5% level of significance. All statistical analyses were performed using SPSS program (standard version 12.0.0, SPSS Inc.).

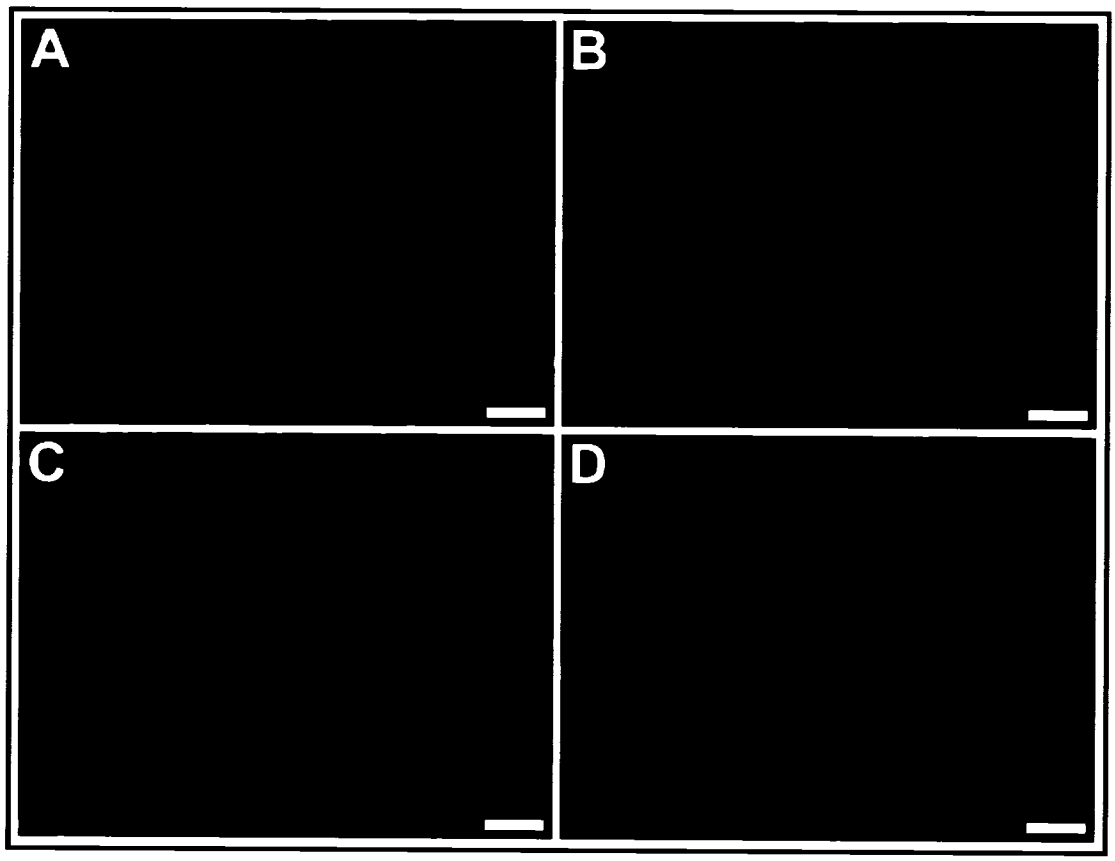

Figure 1: Immunofluorescent identification of satellite cell nuclei in cross sections from pectoralis muscles of both control and Nandrolone birds. **A** and **B** represent a section taken from the pectoralis muscle of a control bird. **C** and **D** represent a section obtained from the pectoralis muscle of a Nandrolone treated bird. **A** and **C** show all nuclei in blue due to labeling by Hoechst, and muscle fibers basal laminae in red labeled by anti-Laminin. **B** and **D** reveal satellite cell nuclei in green due to labeling by anti-Pax7 and basal laminae in red labeled by anti-Laminin. Scale bars = 30 μm.

6.4 Results

6.4.1 Muscle Weights

The weights of pectoralis, iliotibialis cranialis and gastrocnemius externus muscles were significantly ($P < 0.01$) greater in Nandrolone treated birds than in control birds that received saline. The mean weight of pectoralis muscle ± standard error (SE) in the control group was 53.80 ± 1.13 gm, while in the Nandrolone group this value was 65.5 ± 0.93 gm (Fig.2). The weights of iliotibialis and gastrocnemius muscles in the control group were respectively 3.79 ± 0.14 gm and 6.04 ± 0.14 gm, while in the Nandrolone group they were respectively 5.30 ± 0.13 gm and 7.12 ± 0.18 gm (Fig.3).

6.4.2 Size of Fiber profiles

The ellipse minor axis of pectoralis muscle was significantly ($P < 0.01$) larger in the Nandrolone treated group than in the control one. The mean ellipse minor axis ± SE in the Nandrolone group was 39.28 ± 1.56 μm, while in the control group it was 31.59 ± 0.82 μm (Fig. 4).

6.4.3 Giant Fiber Formation

Giant fibers are unusually large fibers that possess circular transverse sectional areas (Cassens et al. 1969, Handel and Stickland 1986). These fibers are scattered throughout the muscle as a very small proportion that is usually less than 1% of total fiber population. In this study, no giant fibers were found in the control

group. However, in the Nandrolone treated birds the mean percent of giant fibers in the total fiber population ± percentage variation was 1.75% ± 0.87. This percent was obtained by examining about 400 fibers from each bird (Fig. 5).

6.4.4 SC Frequency

As evident in Figure 6, the frequency of SCs was significantly ($P < 0.05$) greater in Nandrolone treated birds than in the control ones. The mean frequency of SCs in the control group ± SE was 6.36% ± 0.29. This value was 8.15% ± 0.49 in the Nandrolone treated group.

6.4.5 Number of SC per Unit Length of Fiber

The number of SCs along a mm of fiber was significantly ($P < 0.01$) higher in Nandrolone treated birds than in the control ones. In the control group, the mean number of SCs per mm segment of the fiber ± SE was 5.67 ± 0.32. This value was 8.38 ± 0.40 in the Nandrolone group.

6.4.5 SC Concentration

There was significantly ($P < 0.05$) less surface area of plasmalemma per SC in the Nandrolone treated birds than in the control birds (Fig. 7). The mean surface area per SC ± SE in the control group was 17,602.67 ± 704.47 μm^2. This value decreased to 14,766.25 ± 568.33 in the Nandrolone group. These results indicate that there is a greater concentration of SCs in the Nandrolone treated birds than in the control birds.

6.4.6 Number of Myonuclei per Unit Length of Fiber

The myonuclear number per mm of muscle fiber length was higher in the Nandrolone group, but did not differ significantly ($P = 0.062$) from the control group. The mean myonuclear number per mm of fiber segment ± SE in control group was 81.39 ± 2.21. In the Nandrolone group this mean number ± SE was 92.80 ± 4.45.

6.4.7 Myonuclear Domain Size

The size of the myonuclear domain (volume of cytoplasm per myonucleus) was measured for each group. Similar to the results in chapter 5, the myonuclear domain was significantly ($P < 0.01$) larger in the hypertrophied muscle fibers of Nandrolone treated birds than in those of the control ones (Fig. 8). The mean myonuclear domain size in the control group ± SE was 10,812.04 ± 511.53 μm^3. This mean size value was 14,215.42 ± 488.16 μm^3 in the Nandrolone treated group.

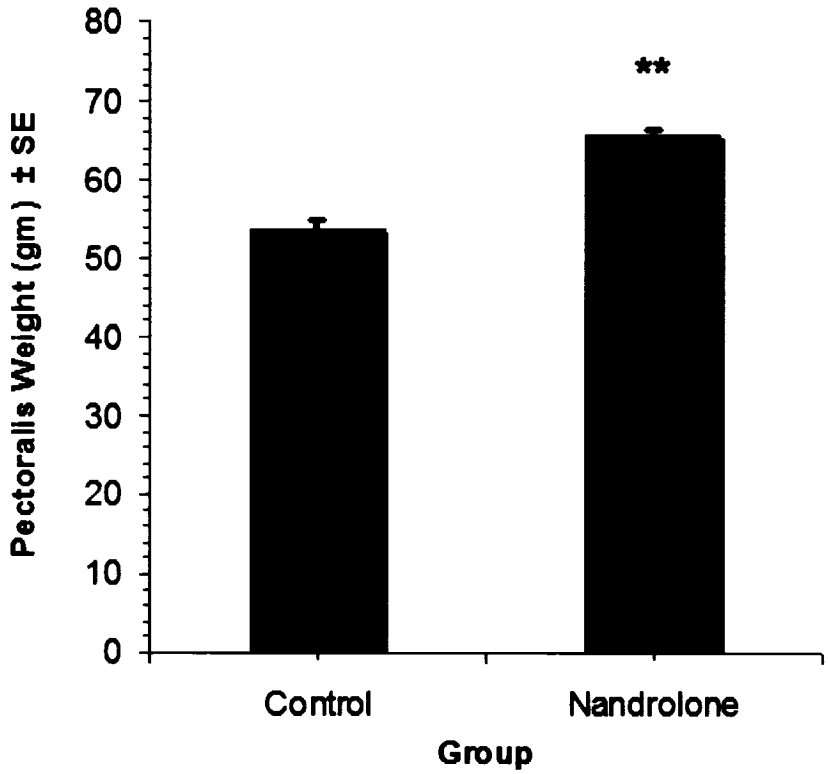

Figure 2: The mean weight of pectoralis muscle for both control and Nandrolone groups. Each column represents the mean pectoralis weight for each group (n=4) in grams (gm) ± standard error (SE). The pectoralis muscle weight was significantly (** = $P < 0.01$) greater in the Nandrolone group.

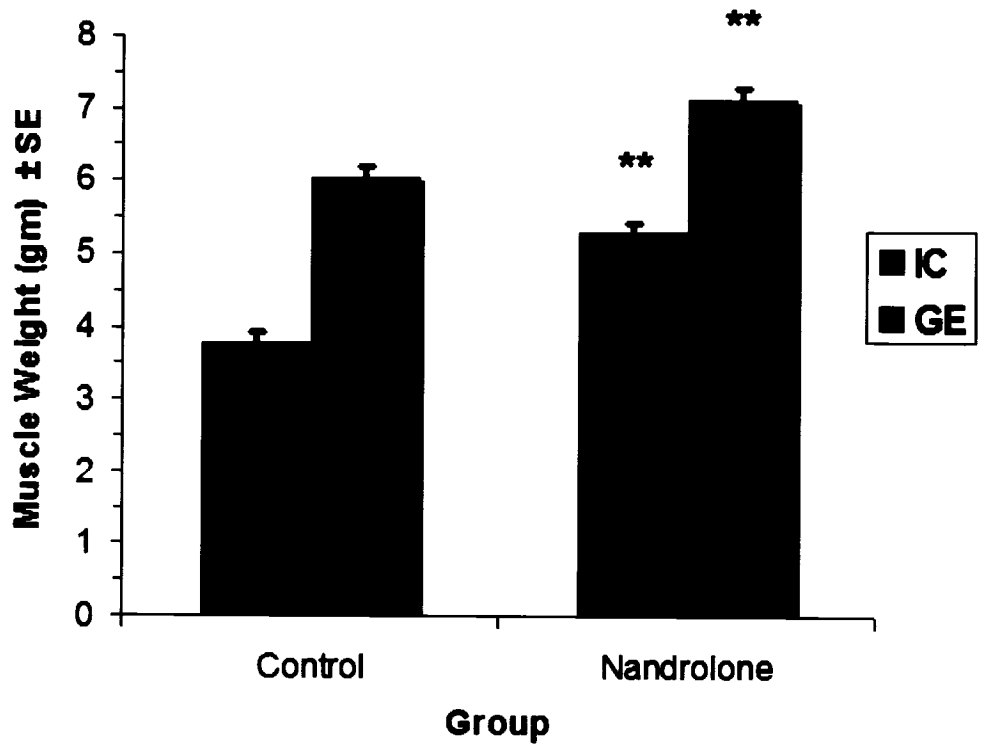

Figure 3: The mean weights of iliotibialis cranialis (IC) and gastrocnemius externus (GE) muscles in both control and Nandrolone groups. The weight of IC and GE muscles was measured for four birds in each group. Each column represents the mean muscle weight for each group in grams (gm) ± standard error (SE). The IC and GE muscle weights were significantly (** = $P < 0.01$) greater in the Nandrolone group.

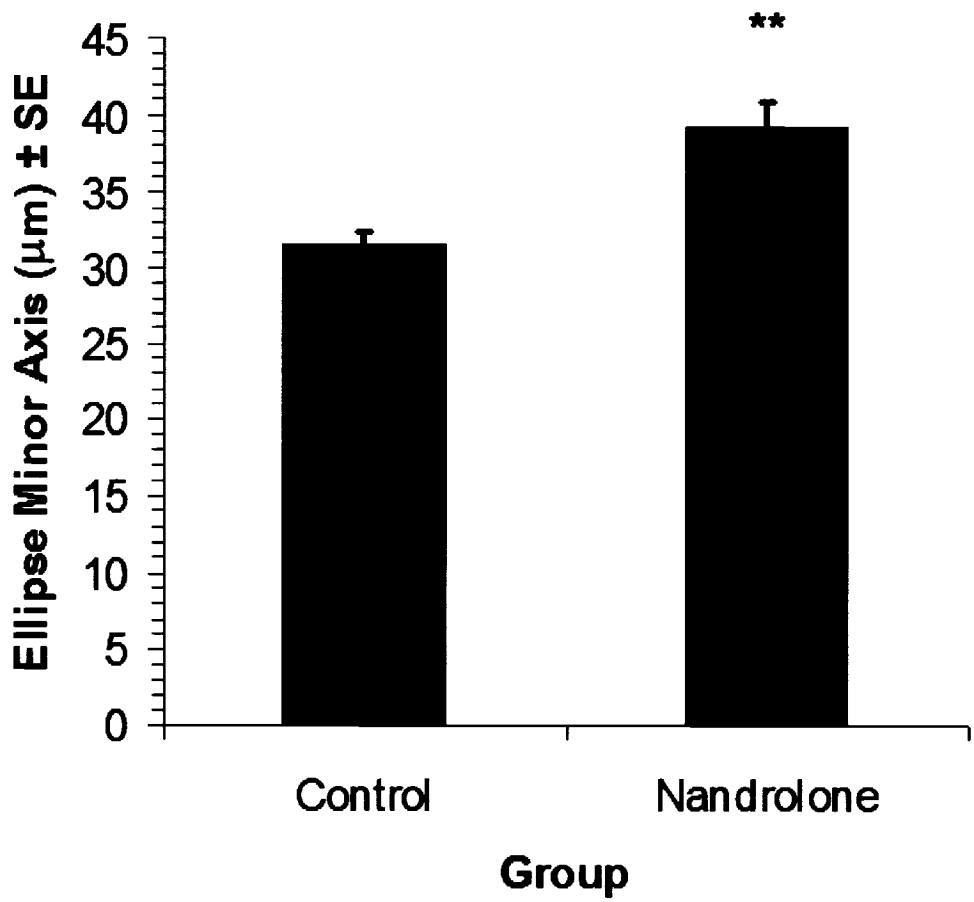

Figure 4: Size of fiber cross sectional profiles in the pectoralis muscle of control and Nandrolone groups. The ellipse minor axis was measured for the pectoralis muscle fibers obtained from four birds in each group. Each column represents the mean ellipse minor axis in micrometers (μm) ± standard error (SE). The minor axis was found to be significantly (** = $P < 0.01$) larger in Nandrolone treated birds.

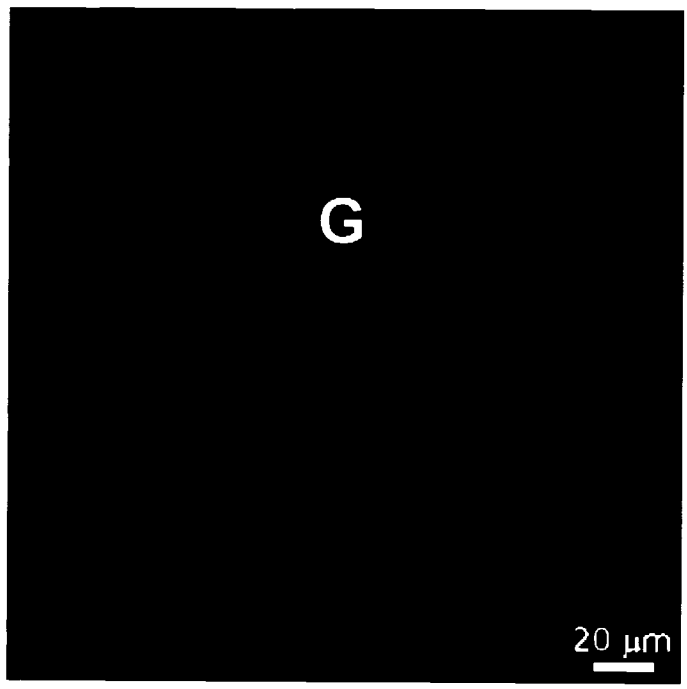

Figure 5: Giant fiber (G) formation in transverse section from chicken pectoralis muscle treated with Nandrolone. The photo shows immunofluorescent labeling by anti-laminin for the muscle fibers basal laminae in transverse section. This section was obtained from chicken pectoralis muscle treated with Nandrolone over a period of four weeks. The photo shows a giant fiber (G) that appears as exceptionally large fiber with circular cross sectional area.

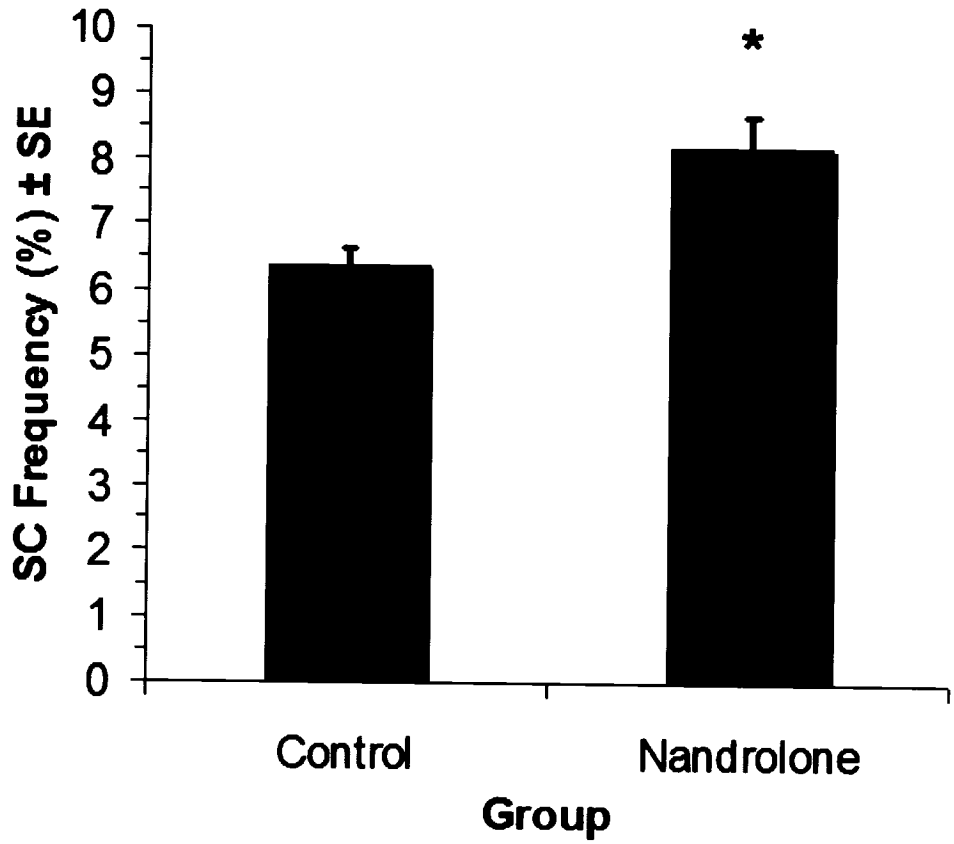

Figure 6: Satellite cell (SC) frequency in the chicken pectoralis muscle of control and Nandrolone groups. The frequency of SCs, (SC nuclei/SC nuclei + myonuclei) x 100%, was obtained for four birds in each group. Numbers of SC nuclei and myonuclei were counted separately for 200 contiguous fiber profiles from each muscle sample. Each column represents the mean SC frequency ± standard error (SE). The frequency was significantly (* = $P < 0.05$) greater in Nandrolone treated chickens.

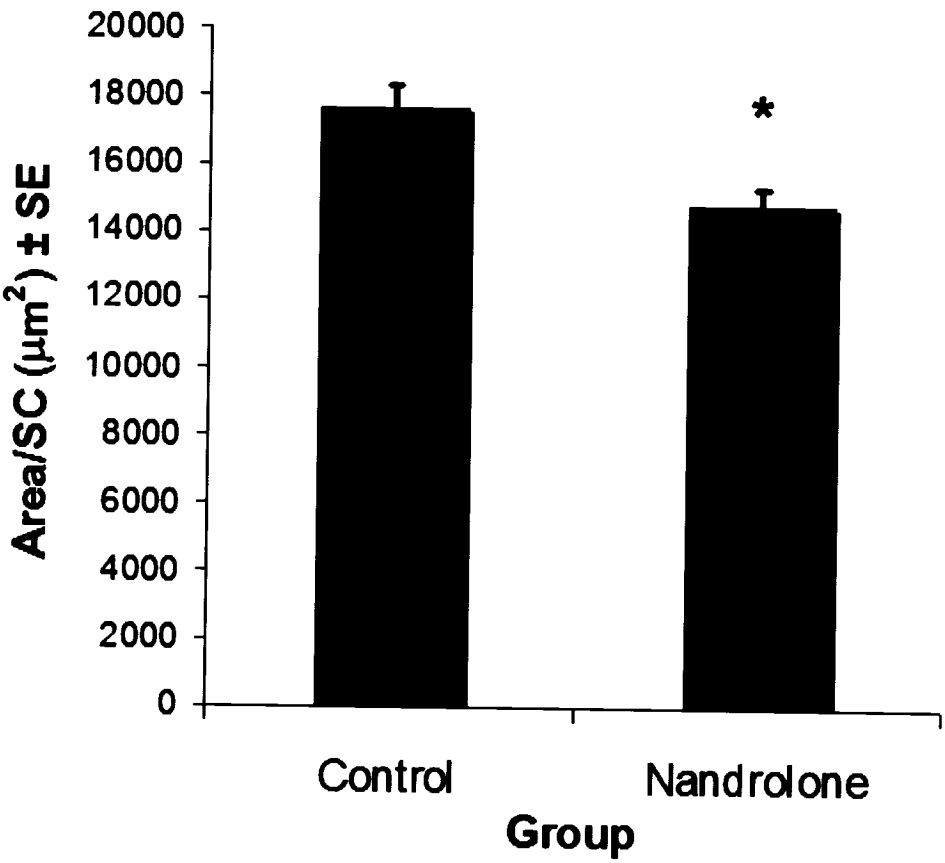

Figure 7: Surface area of sarcolemma per satellite cell (SC) in the pectoralis muscle of control versus Nandrolone treated chickens. The area of sarcolemma (μm^2) for each SC was measured in four birds from each group. Each column represents the mean area of sarcolemma per SC ± standard error (SE). There was significantly (* = $P < 0.05$) less area per SC in the Nandrolone treated chickens than in control. This indicates that SCs were located closer together in Nandrolone birds than in control birds.

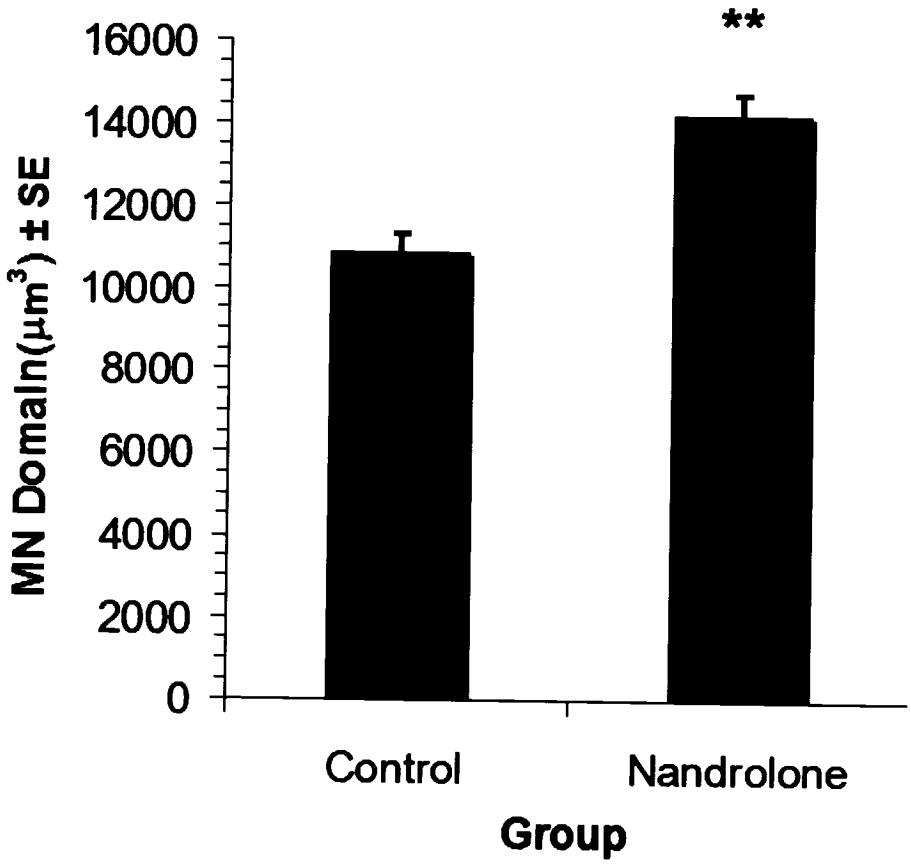

Figure 8: Size of myonuclear domain in the chicken pectoralis muscle of the control and Nandrolone groups. The myonuclear domain size was measured in the pectoralis muscles of four birds from each group. Each column represents the mean myonuclear domain size (μm^3) ± standard error (SE). The domain size was significantly (** = $P < 0.01$) larger in the Nandrolone birds than in the controls.

6.5 Discussion

This study is the first to demonstrate the effects of the anabolic steroid Nandrolone Decanoate on SC distribution. It shows that administration of Nandrolone over a relatively short period of time (4 weeks) can significantly increase the frequency and concentration of SCs within the pectoralis muscle of growing chicken. This result is also accompanied by an increase in fiber size (hypertrophy). Another finding is the observation of giant fibers in the pectoralis of Nandrolone treated birds.

It is already established that AAS boosts weight by increasing the skeletal muscle mass. Bhasin et al. (1996) observed that 10 weeks of testosterone administration (600mg/week) to athletes combined with a strength training program led to significant increase in muscle mass of both triceps brachii and quadriceps muscles. In another study, different dosages of testosterone enanthate were administrated to non-exercising volunteers, and it was found that the increase in quadriceps muscle volume was dose dependent (Bhasin et al. 2001). Kadi et al (1999) reported that the intake of a wide variety of anabolic steroids by power lifter athletes induces an increase in muscle size by both hypertrophy and hyperplasia. Regarding the effects of Nandrolone Decanoate on body mass, many published studies have established that Nandrolone is effective in improving body weight in AIDS patients with and without wasting syndrome (Gold et al. 1996; Gruzdev et al. 1999; Cuerda et al. 2005; Gold et al. 2006). In HIV-patients, Nandrolone has been found to induce a greater lean body mass than testosterone (Shahidi NT 2001, Gold et

al. 2006). Wouter et al. (2004) have also observed the anabolic effects of Nandrolone in bodybuilding athletes. They show that Nandrolone administration at a dose of 200 mg/week for 8 weeks significantly increases body mass and fat free mass in those bodybuilders.

The exact mechanisms by which steroids increase the lean body mass are still poorly understood, because of limited studies that investigate the effects of these steroids at the cellular level. Griggs et al. (1989) found that anabolic steroids increase muscle mass by increasing the rate of muscle protein synthesis. This can be achieved by increasing the number of myonuclei within muscle fibers and/or increasing the activity of preexisting myonuclei. Since proliferation and differentiation of SCs to new myonuclei is a fundamental process for muscle fiber growth, it is expected that anabolic steroids lead to an increase in the number and the activity of SCs within skeletal muscle. Sinha-Hikim and coworkers (2004) have confirmed the expression of androgenic receptors in both SC nuclei and myonuclei. They also suggested that anabolic steroids increase muscle mass by activating both SCs and muscle fibers. The greater number, frequency and concentration of SCs found in Nandrolone chickens of this study support the activation of these SCs due to Nandrolone administration.

The size of myonuclear domain was significantly larger in the hypertrophied fibers of Nandrolone treated chickens than in the fibers of control chickens. This result supports what was found in the previous study of meat producing chickens that represent a natural model of hypertrophy (Chapter 5). As the fiber size and number of myonuclei was significantly increased in the meat producing lines (M and Y lines), still the myonuclear domain size was significantly larger in these lines than in the control line (C line). These findings suggest that the size of myonuclear domain may not be always correlated with the rate of protein synthesis within the muscle.

Giant fibers were observed in the pectoralis of the Nandrolone birds. Giant fibers were first discovered by Wohlfart in 1937, while studying the sartorius muscle from human fetuses and newborns. Giant fibers were subsequently observed in studies of normal stress-resistant and soft pale stress-susceptible porcine skeletal muscles (Cassens et al. 1969; Hendricks et al. 1971; Handel and Stickland 1986). The occurrence of giant fibers has also been described in human subjects suffering from Duchenne muscular dystrophy (Schmalbruch 1982). The giant fibers recognized in this study can be either physiologically hypertrophied normal fibers or abnormally pathological fibers. However, further investigation including an ultrastructural examination of the myofibrillar structure and characteristics of these fibers is warranted.

In conclusion, the present study reveals that Nandrolone administration increases the number of SCs within developing skeletal muscles. This suggests one of the mechanisms by which Nandrolone can induce muscle fiber hypertrophy. This mechanism is through enhancing SC proliferation and differentiation to myonuclei. Further investigations are recommended to determine whether the increase in SC number is dose dependent and if SCs in Nandrolone chickens are more active than in the control.

7.0 CHAPTER

GENERAL DISCUSSION, CONCLUSIONS AND FUTURE DIRECTIONS

This thesis sought to expand the current field of knowledge regarding how muscles grow and regenerate by examining SC distribution, expression markers and activity under different paradigms of growth and hypertrophy. Several new theories and conclusions regarding SCs are proposed based on the findings of this thesis. Different expression patterns of MyoD and Myogenin are found within SCs during maturation, which support the expectation that each of these factors is expressed at a distinctive stage of activated SCs. Also, a greater frequency and a higher concentration of SCs are found at the ends of maturing muscle fibers, and within naturally growing and Nandrolone-induced hypertrophied fibers.

7.1 Pax7 specificity for SCs within chicken pectoralis

It has been confirmed previously that Pax7 is specifically expressed by SCs, and that an antibody against Pax7 can be used to detect SC nuclei within chicken skeletal muscles (Halevy et al. 2004; Allouh and Rosser 2006). Thus, Pax7 antibody was used to identify SC nuclei in this thesis. This thesis demonstrates that 97-98% of nuclei that express Pax7 within chicken pectoralis have been located inside the muscle fibers basal laminae and in close contact to them. This is considered a characteristic location for SC nuclei (Mauro 1961; Wozniak et al. 2005).

The remaining 2-3% of Pax7+ cells were located in the interstitial spaces outside of the basal laminae of the muscle fibers. The presence of these Pax7+ interstitial cells is consistent with the theories that myogenic cells arise from nonmuscle sources (Asakura et al. 2002, Shi and Gary 2006), that satellite cells

migrate between muscle fibers in postnatal muscle (Lipton and Schultz 1979; Hughes and Blau 1990; Morgan et al. 1993) and that myogenic cells of alternate origins reside in the interstitium (Kuang et al. 2006). Myogenic cells in the interstitium have been recently described as either expressing (Schultz et al. 2006) or not expressing (Kuang et al. 2006) Pax7. It is also conceivable that the Pax7 positive interstitial cells in this thesis could be the progenitors of nascent myofibers developing in the interstititum as has been described by a study of chicken anterior latissimus dorsi muscle subjected to weight overload which resulted in hypertrophy (McCormick and Schultz 1992). *De novo* postnatal muscle fiber formation was also described by a study of rapidly growing rat plantaris muscle (Tamaki et al. 2002). However, it is important to note that earlier work has clearly shown that the small fiber profiles in maturing chicken pectoralis are the tapered ends of larger fiber profiles and not nascent fibers (Rosser et al.2000).

7.2 Temporal and spatial distribution of SCs within developing skeletal muscles

The frequency of SCs is significantly decreased during postnatal development until adulthood. This leads to the conclusion that the ability of a muscle to grow and regenerate, and the activity of SCs are significantly reduced as the birds age. This pattern of declination in SC frequency with age has also been noticed in other species such as rodents (Snow 1977; Campion 1984; Shefer et al. 2006) and crayfish (Novotova and Uhrik 1992). The present thesis corroborates the same trend of temporal distribution for SCs within avian skeletal muscle.

Greater frequency and concentration of SCs are detected at the tapered ends of the muscle fibers. This finding indicates that there is a greater capacity for muscle growth and regeneration at fiber ends than other regions along the fiber. During post-natal development, muscle fibers grow in length and girth (Zhang and McLennan 1995; Paul and Rosenthal 2002). The fiber ends are considered the regions for longitudinal growth, where new sarcomeres are added (Swatland 1994; Goldspink 2003). The results of this thesis strongly suggest that SCs have a major role in the longitudinal growth of muscle fibers.

Further investigations are required to address questions arising from the findings of this thesis. Are a greater proportion of the SCs active at the ends than elsewhere along the fibers? To ascertain this, studies involving labeling active SCs with markers such as proliferating cell nuclear antigen (PCNA; Yablonka-Reuveni et al. 1999) or bromodeoxyuridine (BrdU; Mozdziak et al. 1994) would seem appropriate. However, while PCNA has been used to monitor proliferating satellite cells in rodent muscle fibers (Yablonka-Reuveni et al. 1999), recent attempts (unpublished) to use similar approaches in chicken fibers with available antibodies against PCNA have not been successful in this laboratory. Using an antibody against BrdU may be more promising, but this would require extensive experimentation in addition to that performed in this thesis.

To label active SCs with BrdU, white Leghorn chickens could be raised similar to the protocol applied in chapter 4. Time releasing BrdU capsules obtained from Innovative Research (Sarasota, FL) can be implanted subcutaneously when the birds are 62 days post-hatch, since at this age the three different types of fiber profiles are present and a wider range of fiber size can be observed (Rosser et al. 2000). These capsules are designed to deliver a constant dose of 22 μg BrdU/g body mass per day. This has been previously shown to label SCs in quails without altering SC mitosis (Carson and Alway 1996). After two weeks, which is considered a proper period to label all proliferated SCs (Carson and Alway 1996), the birds can be sacrificed and pectoralis muscle samples obtained.

Immunocytochemical methods similar to those used in this thesis would then be applied to the pectoralis sections, using anti-BrdU, anti-Pax7 and anti-laminin antibodies. The percentage of SC nuclei that express BrdU at the fiber ends (ellipse minor axis ≤ 10 μm) can be measured and compared with that of SCs at other regions of the fiber. It is expected that a higher percentage of BrdU+ SCs would be found at the fiber ends. This would mean that greater proportion of SCs are more active at the fiber ends, and that fiber ends are the most active regions of growth within maturing muscle fibers.

Another question arising from this thesis is: Can this finding of high SC numbers at the ends of fibers in post-hatch chicken pectoralis be extrapolated to muscles of postnatal mammals? Studies showing that sarcomeres or newly

synthesized proteins are added to the ends of growing postnatal muscle fibers have utilized primarily mammalian models (Goldspink 2003). Thus, one would anticipate higher concentrations of satellite cells at the ends of mammalian muscle fibers. However, the majority of studies of muscle development utilize mice or rats as experimental models. The fibers within the muscles of these small laboratory rodents generally extend for most of the short muscle length, and attach to a tendon at each end (Burkholder et al. 1994, Sheard et al. 1999). These may not be the best models for studying longitudinal growth in muscle fibers, as most fiber ends are confined to relatively narrow zones near the myotendinous junctions. By comparison, better experimental models may be found in the muscle fibers of less frequently studied larger mammals in which fibers are also relatively short but with the majority of them arranged in series and terminating intrafascicularly (Gaunt and Gans 1992, Sheard et al. 1999) as do those in the avian pectoralis (Gaunt and Gans 1993). These models offer numerous tapered fiber ends that are easily accessed throughout the belly of the muscle. In the present study the chicken pectoralis, which is a muscle rich in fiber ends throughout its belly, has been used to show that SCs are more concentrated and at a greater frequency within growing post-hatch fiber ends.

Muscle fibers within the strap muscles of the hindleg of goats and pigs are serially arranged (Gaunts and Gans 1992). These mammalian muscles could be used to study the distribution of SCs along the fiber length. However, a proper methodology to distinguish the fiber ends on cross sections of these muscles is required first. Within chicken pectoralis, it has been established that the smaller fiber

profiles that express neonatal myosin isoform are the tapered ends of more mature fibers (Rosser et al. 2000; 2002). It is unclear if those serially arranged mammalian fibers would retain neonatal myosin at their fiber ends. It would also need to be shown that the ends of the fibers have smaller cross-sectional profiles in these muscles.

7.3 Patterns of MyoD and Myogenin expression within SC nuclei

The percentages of SCs that express MyoD or Myogenin regulatory factors are found to be significantly greater within post-hatch developing chickens compared to adult chickens. This finding supports the theories that MyoD and Myogenin are expressed within active but not quiescent SCs, since SCs are more active during postnatal developmental periods than in adulthood when they become quiescent (Halevy et al. 2004). Based on this, it is anticipated that antibodies against MyoD and Myogenin factors can be used to distinguish active from quiescent SCs. This has been recently corroborated by using anti-MyoD antibodies to distinguish activated SCs within cultured fibers of extensor digitorum longus muscle of mice (Zammit et al. 2006b). Similar to BrdU antibody, the anti-MyoD antibody could possibly be used to identify active SCs at the fiber ends as has been discussed previously (point 7.2). However, as over 75% of SCs express MyoD after 62 days post-hatch, labeling SCs with MyoD may not reveal differences at fiber ends. Still, it is possible that one might observe a higher percentage of active SCs (Pax7+/MyoD+) at the fiber ends.

During postnatal growth, SCs are expected to be active along the whole fiber length, since muscle fibers grow in length and girth. The fiber size increased in a significant manner ($P < 0.01$) within chicken pectoralis from age 9 to 115 days post-hatch. This increase in girth requires a fair contribution of SCs during this period. This may explain why there is a high percentage of MyoD+ SCs during the post-hatch period.

The percentage of SCs that express MyoD is significantly increased during the post-hatch development period until maturation. Previous cell culture studies have suggested that MyoD expression is required for inhibition of myoblasts proliferation (Sorrentino et al. 1990; Crescenzi et al. 1990; Montarras et al. 1991; Wilson et al. 2003). The temporal pattern of SC distribution showed that the frequency of SCs is significantly reduced during the post-hatch development period (chapter 3). Therefore, it can be expected that as more SCs express MyoD during growth, they cease proliferation and move towards differentiation, which leads to the reduction in SC frequency.

The percentage of SCs that express Myogenin is significantly increased at the early ages of development, and then remained almost steady until maturation where it starts to decrease significantly. Previous cell culture studies showed that Myogenin expression starts at the late differentiation stage of myoblasts, when they start fusing to form myotubes (Montarras et al. 1990; Smith et al. 1994; Yablonka-Reuveni and Paterson 2001). Thus, it is expected at the early stages of post-hatch development

that SC differentiation initially increases rapidly, but then remains at a constant level. After maturation the rate of differentiation is reduced significantly and a greater proportion of SCs become quiescent.

7.4 SC distribution within naturally hypertrophied muscle fibers

The frequency and concentration of SCs have been found to be significantly greater within the pectoralis muscle fibers of meat producing chickens that represent a natural model of hypertrophy than in those of the control chickens. This indicates that natural hypertrophied muscle fibers have a greater ability for growth and regeneration than normal fibers. These findings are similar to those obtained from experimental models of hypertrophy that were studied previously (Winchester et al. 1991; Kadi and Thornell 2000; Sinha-Hikim et al. 2003). However, this thesis is the first study of SC numbers to be conducted on a model where no experimental procedures or enhancing drugs have been required to induce the hypertrophy. The comparable findings between this natural model and experimental models studied previously to induce hypertrophy indicate that most of these experimental models are valid models that can resemble the natural process of hypertrophy.

Unpublished work from this laboratory has compared the chicken pectoralis muscle weight and fiber size between meat producing birds and their control at different ages of post-hatch development. The work shows a similar muscle weight and fiber size between meat and control birds until 14 days post-hatch. This finding raises a question about SC distribution among these birds at that early age. Campion

et al. (1982) have reported a similar fiber diameter within type II fibers of semimembranosus muscle between rapidly growing quails and their control at 4 days post-hatch. They have also reported no significant ($P > 0.05$) difference in SC frequency between these birds and their control at the same age. This thesis shows that as there is no significant ($P = 0.67$) variation in fiber size within the pectoralis of White Leghorn chickens (chapter 3) from age 9 until 23 days post-hatch, there is, also, no significant ($P = 0.25$) difference in the frequency of SCs during this period. Based on these observations, it is hypothesized that no significant difference in SC frequency and concentration is present between meat producing and control birds during early post-hatch growth. However, as all these birds grow there would be a significant decrease in SC frequency. This reduction in SC frequency would be significantly greater within control birds than meat birds, as it has been shown by this thesis. Bird samples of age 14 days post-hatch could be used to test the previous hypothesis. Immunocytochemical procedure would be conducted as in chapter 5 to measure SC frequency among the three bird lines.

Another investigation to confirm the activity of SCs within hypertrophied fibers of meat birds could use the anti-BrdU antibody. The same experimental procedure discussed earlier (point 7.2) could be applied. Since there is a greater frequency and a higher concentration of SCs within meat birds, a higher percentage of BrdU+ SCs is expected within these birds than in the control birds. However, as it has been mentioned in the previous point (7.3) that muscle fibers grow rapidly in

length and girth during post-hatch development, a high percentage of BrdU+ SCs may be detected in both meat and control birds.

7.5 The effects of Nandrolone Decanoate on SCs and morphology of skeletal muscle fibers

This thesis demonstrates that Nandrolone administration increases muscle mass and fiber size. This corroborates previous studies that showed Nandrolone to be an effective drug in promoting body mass (Wouter et al. 2004; Gold et al. 2006) and producing hypertrophy within muscle fibers (Bisschop et al. 1997). Also of greater importance, this thesis is the first work to demonstrate that Nandrolone can significantly increase the frequency and concentration of SCs. This suggests that an enhanced activity of SCs is probably responsible for the increased fiber size and muscle mass within Nandrolone treated birds.

Further study is needed to establish if the effects of Nandrolone on SCs are dose dependent. Answering this question could be useful in the clinical application of Nandrolone for the treatment of muscular dystrophy disorders. Bhasin et al. (2001) have reported a dose dependent increase in quadriceps muscle volume due to testosterone administration. Similarly, a dose dependent increase in chicken pectoralis weight and fiber size due to Nandrolone administration is expected. Since enhanced activity of SCs is one of the main mechanisms in inducing muscle fiber hypertrophy, it is hypothesized that there is a positive dose dependent relationship between Nandrolone administration and SC frequency and concentration.

A similar protocol to that applied in chapter 6 can be used to test this hypothesis. Five groups of growing White Leghorn chicken would receive Nandrolone injections, in addition to a control group that would receive normal saline. The injections would be given in the following range: 10, 20, 30, 40 and 50 mg/week for four weeks. This range is chosen with two dosages below and two above the 30 mg/week dose, which this thesis shows to induce hypertrophy (chapter 6). After that, the birds would be sacrificed and an immunocytochemical procedure similar to that in chapter 6 employed. Lastly, the frequency and concentration of SCs would be compared among these groups to determine if there is a dose dependent increase.

7.6 Requirement of SCs for skeletal muscle hypertrophy

A debate has erupted regarding the necessity of SCs for skeletal muscle hypertrophy. Some researchers have advocated the traditional theory that the addition of SCs is necessary for skeletal muscle hypertrophy (Rosenblatt et al. 1994; Adams et al. 2002). This claim is based on studies that inhibit SC activity and myonuclear addition before applying a hypertrophic stimulus. For example, ablation of SCs by using low doses of gamma irradiation prevented hypertrophy of rodent skeletal muscles (Rosenblatt and Parry 1992; Phelan and Gonyea 1997; Li et al. 2006).

In this thesis, compared to control birds, the frequency and concentration of SCs was found to be significantly greater in both the naturally hypertrophied muscle

fibers of meat birds (chapter 5) and hypertrophied fibers due to Nandrolone injection (chapter 6). Also, an increased number of myonuclei per mm of fiber was present in both natural and Nandrolone hypertrophied fibers compared to their controls. These findings underscore that SCs have an important role during muscle hypertrophy. Thus, certain results of this thesis support the traditional theory that the addition of SCs is necessary for skeletal muscle hypertrophy.

Other researchers, however, indicate that skeletal muscle is capable of hypertrophy without the addition of SCs (Maltin & Delday 1992; Zeman et al. 1994; Lowe and Alway 1999). It is thought that this occurs by enhanced transcription and translation of muscle genes within preexisting myonuclei. This theory is supported by an early study by Fleckman et al. (1978) where the inhibition of DNA replication was not sufficient to prevent skeletal muscle growth and increase in myofibrillar protein content. Later studies using β_2-adrenergic agonists such as clenbuterol or cimaterol in different models including sheep, chickens and rodents showed that administration of these drugs can induce skeletal muscle hypertrophy without any increase in DNA content or myonuclear number (Beermann et al. 1987; Gwartney et al. 1992; Rehfeldt et al. 1994). For example, Maltin and Delday (1992) showed that rats treated with clenbuterol obtained 15% increase in muscle protein content with no change in total DNA or myonuclear number/fiber. More recently, Sharma et al. (1997) reported that mice administered clenbuterol showed a 26% increase in muscle mass with no change in total DNA content.

In this thesis, despite the increased myonuclear numbers, the size of myonuclear domain has also significantly increased within hypertrophied muscle fibers. The expansion of myonuclear domain within these fibers is an indicator of enhanced myonuclear transcriptional and translational activities (Chen et al. 2002) leading to increase in protein content of the domain. Thus, this thesis also supports the theory that the addition of SCs is not necessary for skeletal muscle hypertrophy.

There are some limitations to the experiments supporting both theories. The studies that used gamma irradiation to inhibit SC activity did not provide sufficient data to show that the lack of growth is due to effects only on SCs. Most of these studies focused their analysis just on SCs and ignored the fact that SCs are not the only cells within skeletal muscle that could be affected by irradiation. On the other side, some studies of β_2-adrenergic agonists analyzed the muscle weight and DNA content only. Muscle weight is not an accurate measure, since it includes the connective tissue component of the muscle. DNA content is also an inaccurate measure of myonuclei, since it includes other cell types such as fibroblasts and macrophages. In addition, one of these studies (Beermann et al. 1987) reported an increase in DNA content during late stage of cimatrol-induced hypertrophy. This finding can suggest a role for SCs, even though DNA content is an inaccurate measure.

The view supported by this thesis is that both mechanisms, SC activation and enhanced myonuclear activities, are required for skeletal muscle hypertrophy. It is

anticipated that these two mechanisms are working together to produce muscle fiber hypertrophy, because of greater frequency of SCs and greater size of myonuclear domain that have been found in hypertrophied fibers of this thesis. However, which one of these two mechanisms has the major role in producing the hypertrophy, or whether they work in a coordinated manner, are questions that remain to be answered. Also, the contribution of other cell types to muscle growth and hypertrophy should be considered, as recent studies have implicated some roles for macrophages and endothelial cells in skeletal muscle growth (Takahashi et al. 2002; Tidball and Wehling-Henricks 2007).

7.7 Correlation of myonuclear domain size with the rate of muscle protein synthesis

The theory of myonuclear domain, where each myonucleus controls gene expression in its surrounding sarcoplasm is well established (Cheek et al. 1971; Landing et al. 1974; Pavlath et al. 1989; Allen et al. 1995; Ralston et al. 1997). Researchers have correlated the size of myonuclear domain (volume of cytoplasm per myonucleus) with the rate of protein synthesis and muscle fiber activity in different ways. The first group claims that muscle fibers maintain a relatively fixed myonuclear domain size (Winchester and Gonyea 1992; Allen et al. 1995; Roy et al. 1999). This claim is also supported by researchers proposing that SC addition is obligatory for skeletal muscle growth and hypertrophy (Rosenblatt et al. 1994). This is because they anticipate that addition of SCs is necessary to maintain a fixed

domain size. However, the data supporting this concept is inconsistent with more recent studies (Rosser et al. 2002; Aravamudan et al. 2006).

The second group purports the concept that myonuclear domain size is smaller where there is a higher requirement for protein synthesis and growth (Knizetova et al. 1972; Edgerton and Roy 1991). This claim focuses on the quantity of myonuclei, but not their level of activity. This concept can be refuted by several studies that reported a smaller domain size in atrophied muscle fibers (Allen et al. 1996; 1997; Kasper and Xun 1996a, 1996b; Verheul et al. 2004). It could be argued that these studies did not account for myonuclear apoptosis. However, Wada et al. (2002) have reported that there is no decrease in myonuclear number after long-term denervation of mature mice skeletal muscle.

The inverse relationship between domain size and rate of protein synthesis may be applicable to type I slow oxidative muscle fibers. Type I fibers have a higher rate of protein remodeling than glycolytic type IIb fibers (Kelly et al. 1984; Garlick et al. 1989). This is probably for fiber maintenance rather than hypertrophy or growth. These fibers could have a higher rate of protein breakdown, because they are the main fibers used in routine activities. Hence, it is believed that smaller myonuclear domain (relatively greater concentration of myonuclei) is required within these fibers to allow for more rapid repair and replacement of damaged proteins. On the other hand, during hypertrophy of glycolytic type IIb fibers there is probably a higher rate of

protein synthesis and a lower rate of protein breakdown. This would lead to more protein accretion within the fibers and expansion of the myonuclear domain.

The findings of this thesis show that despite the increase in myonuclear number, the size of myonuclear domain is significantly increased in both natural and Nandrolone induced hypertrophied fibers compared to their controls. That is the size of myonuclear domain is dynamic and adaptable to the surrounding situations. This thesis suggests that myonuclear domain size may not be a strong indicator for the rate of protein synthesis or level of activity within muscle fibers. Undoubtedly, the size of myonuclear domain is controlled by many factors, including fiber type, rate of protein synthesis, rate of protein breakdown and SC differentiation.

7.8 Can Nandrolone Decanoate induce skeletal muscle hyperplasia?

The two types of muscle growth, hypertrophy (increase in fiber size) and hyperplasia (increase in fiber number), are considered a major area of research interest by muscle biologists (Veggetti et al. 1990; Antonio and Gonyea 1993; Kadi et al. 1999). Hypertrophy is an adaptive process of muscle growth that occurs more frequently in response to growth stimuli than hyperplasia (Kadi and Thornell 2000; Hawke and Gary 2001). Hypertrophy is a reversible process, because after cessation of growth stimuli, such as during immobilization or discontinued use of anabolic steroids, skeletal muscle mass decreases due to substantial depression in muscle protein synthesis (Rennie et al. 2004). On the other hand, hyperplasia is a less common but more spectacular process of muscle growth, since it involves formation

of new muscle fibers (Antonio and Gonyea 1993). It has been reported that muscle fiber number is constant after birth (Gollinck et al. 1981; 1983). However, there is abundant evidence that hyperplasia can occur during postnatal growth and in adulthood (Alway et al. 1989a; 1990; Giddings and Gonyea 1992; Sjostrom et al. 1992; Antonio and Gonyea 1994).

The administration of Nandrolone in the current thesis resulted in a statistically significant ($P < 0.01$) increase in the number of SCs. It is well established that SCs act through both hypertrophy and hyperplasia to produce skeletal muscle growth (Hawke and Gary 2001; Zammit et al. 2006a). Can the administration of Nandrolone decanoate significantly induce skeletal muscle hyperplasia? Answering this question would be helpful in the use of Nandrolone for the treatment of patients with muscle dystrophy, since the major objective in these patients is to induce a better improved muscle growth by producing new healthy fibers rather than increasing the size of preexisting often pathological fibers.

Further investigations are required to test the hypothesis that Nandrolone Decanoate induces muscle fiber hyperplasia within both slow and fast-twitch skeletal muscles. A similar experimental procedure for the raising and injection of the chickens as in chapter 6 can be applied. Samples from the pectoralis muscle and the whole anterior latissimus dorsi (ALD) muscle would be excised from both a Nandrolone treated group and a control group. Chicken pectoralis is a large muscle composed almost entirely of glycolytic fast-twitch type IIb fibers. These fibers are

arranged in series that overlap each other from the origin to the insertion of the muscle (Rosser et al. 2000; 2002). ALD is a small, slow-tonic muscle with parallel fibered structure in which all fibers extend from the origin to the insertion of the muscle (Alway et al. 1989a). Similar tissue harvesting and immunocytochemical protocols as elsewhere in the thesis would be applied.

The first investigation would be conducted on the pectoralis muscle sections, and would use antibodies against laminin and leu 19. Leu 19 is a cell-cell recognition molecule expressed during early stages of fiber formation (Schubert et al. 1989). Using the leu 19 antibody it should be possible to identify the newly formed fibers within the pectoralis sections. After that, the percentage of these newly formed fibers of the total fiber population could be obtained. The percentage of newly formed fibers within pectoralis muscles of Nandrolone group would be compared to those of the control group. A higher percentage of newly formed fibers within the pectoralis of Nandrolone treated birds compared to control birds would indicate that Nandrolone induces hyperplasia.

The second investigation would use ALD sections from Nandrolone and control groups. An immunocytochemical procedure with anti-laminin antibody would be used to determine the fiber boundaries in ALD midbelly sections. The cross-sectional area of fiber profiles could be measured using available software programs. After that, the number of fibers within each muscle would be determined using the following formula: total fiber number = muscle cross-sectional area / mean

fiber area (Alway et al. 1989b). The Nandrolone and control groups would be compared for differences in total fiber counts to quantify hyperplasia. It is important to note that this count procedure cannot be applied to pectoralis muscle, because the pectoralis fibers overlap, and the complex shape and size of the pectoralis make it impossible to measure total muscle cross-sectional area.

7.9 Occurrence of giant fibers in Nandrolone treated muscles

Giant fibers are exceptionally large fibers that have circular transverse sectional areas, and are scattered throughout the muscle in very small numbers. These fibers were first described by Wohlfart in 1937, and he called them B fibers. Unfortunately, the exact nature of these fibers is still not fully understood due to insufficient research.

There is disagreement in the literature on whether these giant fibers occur pathologically or not. Wohlfart stated they are not, despite their presence in some forms of muscular atrophy (Wohlfart 1949). Also, giant fibers have been observed in normal developing human muscles (Dubowitz 1965a) and in fetal muscles of guinea pigs (Dubowitz 1965b). Additional studies have reported the presence of giant fibers in the muscles of low quality, stress susceptible pigs, and have suggested these fibers are associated with certain abnormal conditions (Cooper et al. 1969; Dutson et al. 1978). Conversely, other studies have reported similar percentages of giant fibers in the muscles of healthy normal pigs (Hendricks et al. 1971; Handel and Stickland 1986).

Fibers with histological appearance similar to giant fibers have been found in muscle dystrophy disorders (Shafiq et al. 1969; Schmalbruch 1982). However, in dystrophy disorders these fibers exist in large groups or sometimes in whole bundles, while giant B fibers are scattered throughout the muscle and form a small proportion of the total fiber population (Cassens et al. 1969). Also, muscle dystrophy disorders are invariably associated with degenerative changes (Shafiq et al. 1969; Schmalbruch 1982). Handel and Stickland (1986) have reported no degenerative changes in giant fibers of healthy muscle compared to the large fibers encountered in muscular dystrophies.

The giant fibers observed in the Nandrolone treated chickens in this thesis are not likely associated with any pathological condition. They were scattered throughout the histological sections and represented a small proportion of the total fiber population. Subsequently, ultrastructural investigation could help determine the exact nature and any potential structural abnormalities these fibers may have. Also, previous study has reported that damaged fibers within chicken pectoralis regenerate by initially expressing the neonatal MyHC isoform (Cerny and Bandman 1987). Thus, using an antibody against neonatal myosin and immunocytochemical techniques, giant fibers could be examined for the presence of neonatal MyHC. The neonatal MyHC within giant fibers would be an indicator of an abnormal condition.

SUMMARY

This thesis elucidates new findings regarding SC distribution within normal and hypertrophied skeletal muscles during post-hatch development. It also reveals new information about the *in vivo* patterns of MyoD and Myogenin expression within SC nuclei during post-hatch development. As the frequency of SCs decreases significantly with age, the tapered fiber ends retain the highest SC frequency and concentration. The fiber ends are considered the active regions for longitudinal growth within skeletal muscles. Many studies have examined the expression of MyoD and Myogenin *in vitro*, and suggest a specific pattern of expression for each of these factors. This thesis examines the *in vivo* expression patterns for these factors during growth. The percentage of MyoD+ SCs increases significantly during post-hatch development, while the percentage of Myogenin+ SCs remains steady. After maturation, however, the frequencies of MyoD+ and Myogenin+ SCs decrease significantly as the ability of the muscle to grow is reduced and more SCs become quiescent. SC distribution is also obtained by studying meat producing chickens that represent a natural model of skeletal muscle hypertrophy. Significantly higher frequencies and concentrations of SCs are found within naturally hypertrophied fibers compared to their controls. Also a greater number of myonuclei per unit length of the fiber is detected in hypertrophied muscles. The steroid Nandrolone Decanoate is then used to induce skeletal muscle hypertrophy within normal chickens. A significant increase in fiber size results from Nandrolone administration. Nandrolone also leads to higher frequency and concentration of SCs and greater number of myonuclei per unit length of the fiber within growing muscles.

All these results indicate the importance of SCs for skeletal muscle growth and hypertrophy. SC frequency and concentration are always greater when a higher capacity for growth is present, such as during development and hypertrophy. The greater number of myonuclei detected per unit length within all models of hypertrophy is indicative of greater numbers of SCs, since SCs are the primary if not only source of myonuclei after birth. In addition to greater SC number, an increase in the activity of SCs is probably responsible for the greater growth capacity within developing and hypertrophying muscles. This is corroborated by the higher percentages of MyoD+ and Myogenin+ SCs during postnatal development, since MyoD and Myogenin are expressed only within active SCs.

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Appendix A

Chemical Solutions for Immunocytochemistry

Stock Solutions stored at -20^{O}C

1. Phosphate Buffer Saline (PBS) 10x:
   For 400 ml: 100ml Monobasic + 300 ml Dibasic
   Monobasic Sodium Phosphate (NaH_2PO_4) -
   2.76g in 100ml dH_2O
   Dibasic Sodium Phosphate (Na_2HPO_4) -
   8.52g in 300 ml dH_2O
   Add together and pH to 7.2
   Add 35.06g NaCl

2. Phosphate Buffer (PB) 10x:
   For 400ml: same as PBS except do not add saline (NaCl)

3. 5mM EDTA in PBS (1x):
   For 100ml: 0.186g EDTA in 100ml PBS (1x)
   pH to 7.2

4. 10% BSA:
   For 20ml: 2g BSA in 20ml dH_2O
   Mix thoroughly & leave for 4 hours
   Aliquot in 5 tubes (5ml each) and store at -20^{O}C

Stock Solutions stored at +4°C

5. PBS (1x):
   For 1L: 100 ml PBS (10x) and 900ml dH_2O

6. PB (1x):
   For 1L: 100 ml PB (10x) and 900 ml dH_2O

7. 4% Buffered Formalin:
   for 100 ml: 4ml formaldehyde and 96 ml PBS (1x)
   or : 4g paraformaldehyde to 100 ml warm PBS
   add 1N NaOH until solution clears
   let cool to room temperature & then filter.

Appendix B

Chemical Solutions for Western Analysis

1. Extracting (Lysis) Buffer:
   (For 10 ml solution)

   0.5 ml Tris-HCl (1M) pH 7.5
   1.5 ml NaCl (1M), (made 0.584g/10ml dH_2O)
   50 µl NP-40
   8.0 ml dH_2O
   * mix & store at 4^OC fridge. Just before use take the volume you need (usually 1ml), and add to it the following;

   1µl/ml DTT (1M), stored in -20^OC freezer, (0.772g/5ml dH_2O)
   5µl/ml PMSF (0.2M), stored at -20^OC, (0.175g/5ml isopropanol)
   1µl/ml leupeptin (10mg/ml), stored at -20^OC, (1mg/100µl dH_2O)
   10µl/ml pepstatin (1mg/ml), stored at -20^OC, (1mg/ml Ethol90%:acetic acid10%)
   2µl/ml aprotinin (10mg/ml), stored at -20^OC, (1mg/100µl dH_2O)
   1µl/ml NaF (1M), stored at -20^OC, (0.21g/5ml dH_2O)
   1µl/ml Na_3Vo_4 (1M), stored at -20^OC, (0.184g/1ml dH_2O)
   * For 0.2g of sample 1ml lysis buffer is needed (1:5 ratio of sample:buffer).

2. 30% Acrylamide Mix:
   Acrylamide: 29.2g/100ml
   N'N'Bis-methylene-acrylamide: 0.8g/100ml

   *Store at 4^OC fridge in dark for 30 days max.
   (Cover the bottle with tin fowl)

3. 10% SDS (@ room temp.):
   10g SDS in 90ml dH_2O, and then add to 100ml.

4. 1.5M Tris-HCl, pH 8.8 (separating gel buffer):
   27.23g Trisbase (18.15g/100ml)
   80ml dH_2O
   adjust to pH 8.8 with 6N HCl, & then bring to 150ml.

5. 0.5M Tris-HCl, pH 6.8 (stacking gel buffer):
   6g Trisbase
   60ml dH_2O
   adjust to pH 6.8 with 6N HCl, & then bring to 100ml

6. 10X Running (Tank) Buffer:
   24.24g Trisbase
   115.2g Glycine
   8g SDS
   Dissolve & bring to 800ml dH_2O, do <u>not</u> adjust pH.
   If precipitated warm to room temp. before use.
   * For each run: dilute 80ml of 10X stock with 720ml dH_2O
   & mix thoroughly before use.

7. 10% APS (Fresh Daily or in fridge):
   100mg ammonium persulfate in 1ml dH_2O.

Appendix C

Technical difficulties and resolutions

Weak Reactivity of anti-Pax7

Pax7 antibody was suggested to be used at a dilution range of 1:5 – 1:25. This dilution of Pax7 antibody was increased to 1:100 by increasing its incubation period from 1 hour to overnight (12-14 hours) at 4°C.

Fading of immunofluorescence staining

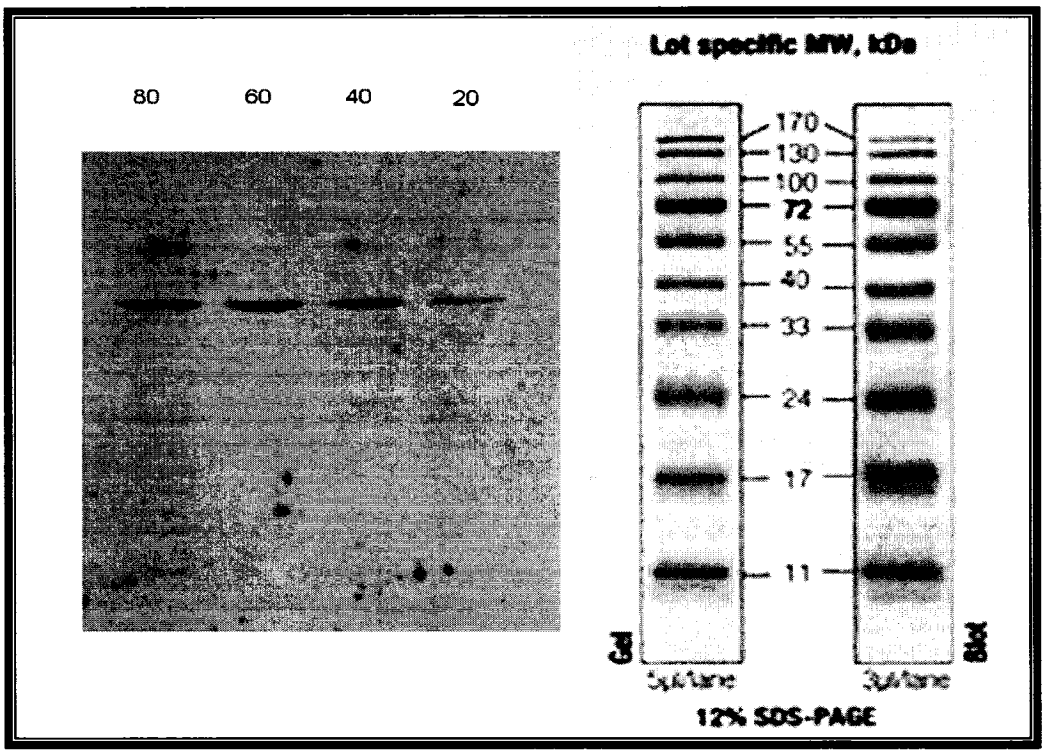
The immunofluorescence labeling was quickly fading when placed under the fluorescent microscope. This problem was solved by; 1) increasing the concentration of the secondary antibodies from 1:500 to 1:200, 2) adding a preservative material (p-Phenylenediamine, Sigma Chemical Co.) to the mounting medium and 3) reducing the power of epifluorescence by half.

Weak reactivity of anti-Myogenin antibody

Anti-Myogenin antibody had a weak reactivity with tissue section. It had been used, as recommended, at a dilution of 1:25. The reactivity was increased by 1) treating the tissue section with 60% acetone for 10 min before applying the primary antibodies and 2) by increasing the incubation period of the primaries to overnight (12-14 hours).

Appendix D

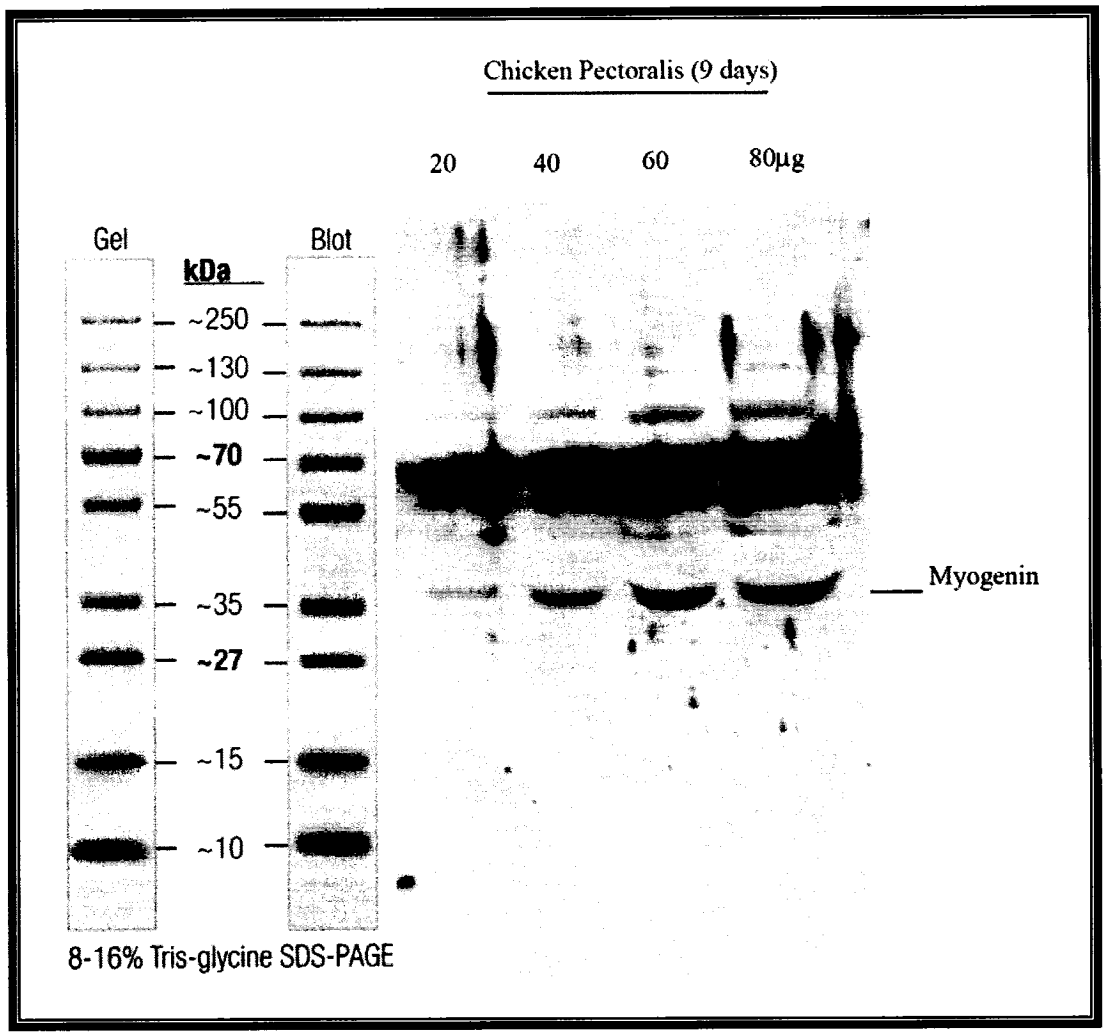
MyoD Western Analysis



A whole membrane photo for MyoD Western analysis of age 9 days post-hatch chicken pectoralis. The numbers above the membrane indicate the amount of protein extract placed in each lane in μg. The molecular marker guide, on the right half of the figure, is placed as it was presented by the manufacturer (Fermentas Life Sciences, Ontario, Canada).

Appendix E

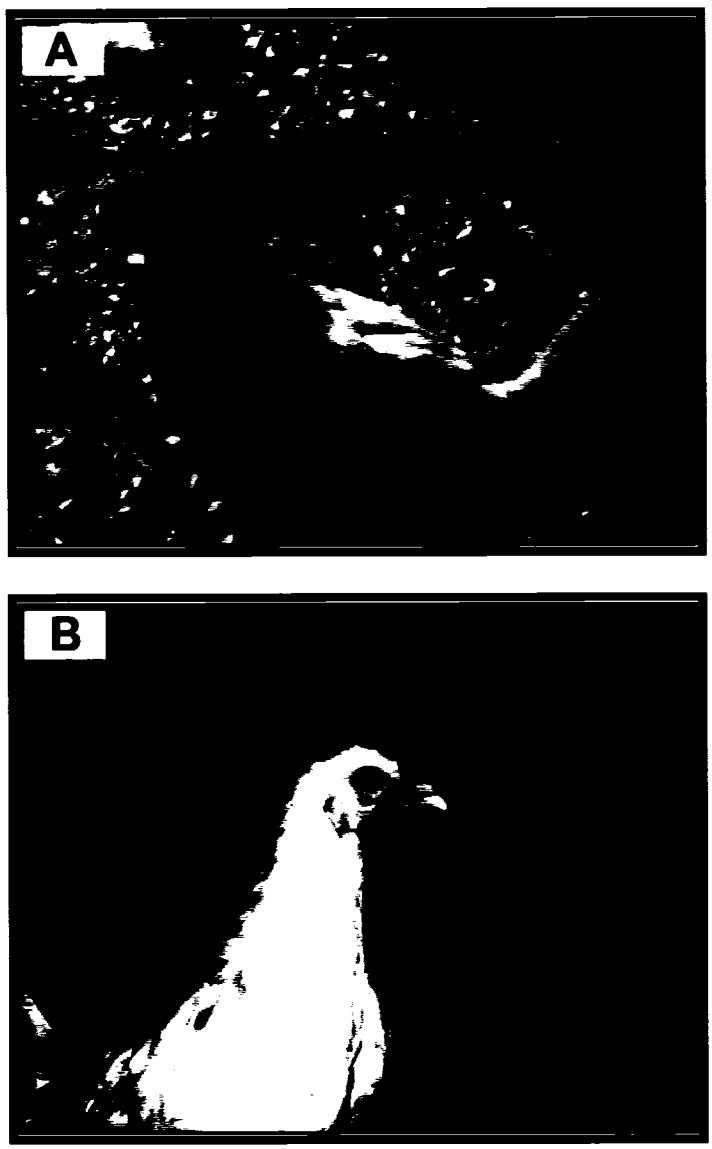
Myogenin Western Analysis



A whole membrane photo for Myogenin Western analysis of age 9 days post-hatch chicken pectoralis. The numbers above the membrane indicate the amount of protein extract placed in each lane in µg. The dark band detected at 70 kDa is probably due to Myogenin dimerization. The molecular marker guide, on the left half of the figure, is placed as it was presented by the manufacturer (Fermentas Life Sciences, Ontario, Canada).

Appendix F

Control vs. Nandrolone Chicken Photos



Photos were taken after three weeks of starting the injections. (**A**) Shows a bird from the control group that received saline injections. (**B**) Shows a bird from the Nandrolone group that received Nandrolone injections. The Nandrolone birds show very well developed combs and wattles, which are considered a secondary sexual characteristic of roosters. Some of the feathers on the Nandrolone birds (B) are labeled with a red color to distinguish these birds from the controls.