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Satellite Cell Proliferation and Differentiation During Postnatal Growth of Porcine Skeletal Muscle

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SATELLITE CELL PROLIFERATION AND DIFFERENTIATION DURING POSTNATAL GROWTH OF PORCINE SKELETAL MUSCLE

By

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ABSTRACT

SATELLITE CELL PROLIFERATION AND DIFFERENTIATION DURING POSTNATAL GROWTH OF PORCINE SKELETAL MUSCLE

By

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DNA accretion is essential to skeletal muscle growth and is a result of satellite cell proliferation, differentiation and fusion with existing muscle fibers. The objective of this study was to quantify populations of proliferating and differentiating satellite cells throughout porcine skeletal muscle growth. Satellite cells were isolated from the *semitendinosus* (ST) muscle of pigs at 1, 7, 14, and 21 weeks of age, separated from cellular debris and immunostained. Satellite cells were quantified by positive staining for a muscle-specific neural cell adhesion molecule (NCAM). Antibodies against proliferating cell nuclear antigen (PCNA) and myogenin were employed to quantify proliferating and differentiating satellite cells, respectively. Increases in ST muscle weight were closely paralleled by increases in total DNA and protein. The proportion of satellite cells to non-myogenic cells as well as the rate of differentiation decreased from 1 to 7 weeks. A modest decline in the percentage of proliferating satellite cells occurred from 1 to 21 weeks with a large proportion (>75%) remaining proliferative at 21 weeks. These results indicate that a high percentage of satellite cells remain proliferative throughout postnatal growth and the rate of differentiation may be a critical regulatory point for skeletal muscle growth.

This thesis is dedicated to my parents who have always believed in me and have given me every opportunity to be the best I could be. This is also for Kristin who is my inspiration and has been there with love and encouragement every step of the way.

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LIST OF ABBREVIATIONS

^{3}H-Thymidine: Tritiated thymidine

ACTH: Adrenocorticotropic hormone

BrdU: 5-bromo-2'-deoxyuridine

CK: Creatine kinase

CME: Crushed muscle extract

CNR: Cytoplasmic volume to nucleus ratio

DAPI: 4',6-diamidino-2-phylindole

DNA: deoxyribonucleic acid

EGF: Epidermal growth factor

ELISA: Enzyme-linked immunosorbent assay

FBS: Fetal bovine serum

FGF-2: Fibroblast growth factor-2 or basic fibroblast growth factor

FITC: Fluorescein isothiocyanate

HGF: Hepatocyte growth factor

HM: Heavy muscled

IGFBP: Insulin-like growth factor binding protein

IGF-I: Insulin-like growth factor I

IGFR-1: Insulin-like growth factor receptor-1

LD: *longissimus dorsi*

LM: Light muscled

MAPK: Mitogen-activated protein kinase

M-CSF: Macrophage colony-stimulating factor

MEM: Minimum essential medium

MHC: Myosin heavy chain

mRNA: Messenger ribonucleic acid

NCAM: Neural cell adhesion molecule

PBS: Phosphate buffered saline

PCNA: Proliferating cell nuclear antigen

PDGF: Platelet-derived growth factor

RNA: Ribonucleic acid

SEM: Standard error of the mean

ST: *semitendinosus*

TRITC: Tetramethyl rhodamine isothiocyanate

INTRODUCTION

Elucidation of the cellular mechanisms governing skeletal muscle growth will be beneficial for development of strategies to increase the efficiency of lean tissue growth in food animal species. Increases in efficiency of animal growth would also lessen the burden of these production systems on financial and environmental resources. Furthermore, such discoveries may provide insight into the treatment of human diseases such as muscular dystrophy.

Skeletal muscle growth occurs through hyperplasia (an increase in muscle fiber number) and hypertrophy (an increase in muscle fiber size). Since the majority of hyperplasic growth occurs before birth (Stickland et al., 1975; Wigmore and Stickland, 1982), optimization of postnatal skeletal muscle growth must occur through manipulation of mechanisms regulating muscle hypertrophy. Skeletal muscle hypertrophy is accompanied by DNA accretion in individual fibers and increases in the protein synthesis capacity of the muscle (Powell and Aberle, 1975; Cheek et al., 1970, 1971). Satellite cells, first discovered by Alexander Mauro in 1961, are the sole source of DNA that is added during postnatal skeletal muscle hypertrophy (Schultz and McCormick, 1994). Satellite cells develop as a discrete myogenic cell type in the later stages of embryonic development (Stockdale, 1992), but do not terminally differentiate and fuse with developing muscle fibers as do embryonic myoblasts. Since muscle fiber nuclei are incapable of further DNA synthesis (Stockdale and Holtzer, 1961), satellite cells function to provide DNA for the postnatal growth and regeneration of muscle fibers (Campion, 1984). Satellite cells proliferate to produce a population of

daughter cells, a subset of which differentiate and fuse with existing muscle fibers to facilitate muscle hypertrophy. The remaining undifferentiated cells continue to proliferate or become quiescent to be available for future growth or regeneration (Moss and LeBlond, 1971).

Satellite cells exhibit a high degree of proliferation at birth, but the percentage of nuclei in skeletal muscle that synthesize DNA declines in the later stages of life as these cells become mitotically quiescent (Schultz and Lipton, 1982; Mulvaney et al., 1988; Mozdziak et al., 1994). Although satellite cells have been shown to differentiate and fuse (Stockdale and Holtzer, 1961; Moss and LeBlond, 1971), it is unknown if the *in vivo* rate of differentiation changes throughout postnatal growth. Characterization of these indices of satellite cell activity will provide insight into the mechanisms of DNA accretion and muscle hypertrophy as well as the age at which application of growth promoting agents to growing animals may be the most beneficial.

A variety of hormones and growth factors influence satellite cell activity and muscle hypertrophy (Allen and Rankin, 1990; Dodson et al., 1996), yet the interactions of these growth factors and the mechanisms by which they affect myogenesis *in vivo* are unknown. Furthermore, the levels of these growth factors present in muscle throughout postnatal life and their effects on growth are unknown. Therefore, identification and characterization of the pertinent growth factors regulating skeletal muscle growth may lead to advances in the development of new strategies to optimize skeletal muscle growth.

CHAPTER 1

LITERATURE REVIEW

Satellite Cells and Skeletal Muscle Growth

Since muscle fiber number is determined prenatally and remains constant throughout life, postnatal growth occurs by hypertrophy of existing muscle fibers (Stickland et al., 1975; Wigmore and Stickland, 1982, 1983). A number of studies have shown that postnatal muscle hypertrophy is dependent upon DNA accretion. Powell and Aberle (1975) established this association through comparison of heavy muscled (HM) and light muscled (LM) growing pigs. Heavy muscled pigs exhibited a greater *biceps femoris* muscle mass, as well as a greater total DNA content than LM pigs. These data indicate that greater rates of hypertrophy of muscle are dependent upon accretion of DNA. These findings are corroborated by studies of Cheek et al. (1970, 1971), who concluded that a muscle nucleus can accommodate a fixed cytoplasmic volume. In other words, there is a fixed ratio of DNA to protein. Taken together, these studies demonstrate that DNA accretion is necessary to increase the protein synthetic capacity of muscle leading to fiber hypertrophy and ultimately muscle growth.

It is generally accepted that nuclei situated under the sarcolemma of muscle fibers, termed myonuclei, lose the ability to synthesize DNA. In a study by Stockdale and Holtzer (1961), multinucleated myotubes were examined both *in vitro* and *in vivo* to determine the source of incorporated nuclei. These authors observed that a 30-minute exposure of embryonic chick muscle to tritiated

thymidine (^{3}H-Thymidine) labeled only mononucleated cells that were situated outside of the sarcolemma, indicating that only these nuclei were synthesizing DNA. Identical treatment of myotube cultures *in vitro* failed to label any nuclei that had been incorporated into a myotube. However, mixed myoblast/myotube cultures pulse labeled for 30 minutes, washed and kept in culture for an additional 4 days did produce labeled nuclei within myotubes, demonstrating fusion of mononucleated cells that had been synthesizing DNA at the time of ^{3}H-Thymidine exposure into myotubes. This study demonstrated that myotube formation occurs through fusion of multiple mononucleated cells, not through mitotic or amitotic division of nuclei already located in the myotubes. In a similar experiment, nuclei incorporated into a myotube failed to incorporate ^{3}H-Thymidine yet myotubes were still able to accumulate DNA to facilitate a regeneration response (Snow, 1978). These studies demonstrate that DNA accretion of skeletal muscle fibers occurs through incorporation of mononucleated cells that reside outside of the sarcolemma.

At birth, mononucleated cells continue to reside beneath the basement membrane of individual muscle fibers yet remain outside of the sarcolemma (Stockdale, 1992; Mauro, 1961). These mononucleated cells were termed satellite cells and were first discovered in the *tibialis anticus* muscle of the frog by Alexander Mauro in 1961. Satellite cells consist of a heterochromatic nucleus, scant cytoplasm and few organelles (Mauro, 1961; White and Esser, 1989). The developmental time point in which satellite cells and embryonic myoblasts diverge and become separate myogenic cell lines is unknown (Campion, 1984).

One hypothesis postulates that satellite cells are remnants of embryonic myoblasts that do not terminally differentiate and are carried forward and encased within the basement membrane. Another plausible hypothesis speculates that satellite cells and embryonic myoblasts diverge as separate myogenic lineages when pluoropotent stem cells undergo determination (White and Esser, 1989; Schultz and McCormick, 1994).

Satellite cells increase the DNA content of existing muscle fibers through proliferation, followed by differentiation and fusion of these daughter cells with the fibers. In a seminal study by Moss and LeBlond (1971), young Sherman rats (14-17 days of age) received a single intraperitoneal injection of ^{3}H-Thymidine, and were sacrificed 1 to 72 hours after injection. Transverse sections of *tibialis anterior* muscle were analyzed to quantify those nuclei that incorporated the label as well as the position of those nuclei. Satellite cells were found to be the only cell type to incorporate the label at the earliest time points, the percentage of which increased to 10 hours post-injection. An increase in labeled myonuclei was observed at 24 and 48 hours, while the percentage of labeled satellite cells decreased at these time points. Results of Allbrook et al. (1971), corroborate the above findings through quantification of the degree to which satellite cells incorporate ^{3}H-Thymidine. One hour after injection, satellite cells exhibited a high degree of labeling (4-8 silver grains per nucleus). At 24 hours post-injection the average number of silver grains per satellite cell nucleus is reduced (1-4 silver grains per nucleus), with a concommitant increase in the number of labeled myonuclei. These data provide conclusive evidence that postnatal DNA

accretion occurs through proliferation of satellite cells, followed by the differentiation and fusion of daughter cells with existing muscle fibers. Furthermore, daughter cells that do not terminally differentiate provide for a "reserve" population for future cycles of proliferation and differentiation.

Satellite cells are usually mitotically quiescent in mature uninjured muscle (White and Esser, 1989), yet can be recruited into the cell cycle following a wide range of events including injury, denervation, exercise, overuse (Schultz and McCormick, 1994) or during growth (Moss and Leblond, 1970). Satellite cell activity is regulated in part at the transition from quiescence to proliferation (Figure 1.1.). For example, treatment of quiescent satellite cells *in vitro* with hepatocyte growth factor (HGF) causes satellite cells to have a shortened lag phase of growth when compared to control cultures. This indicates a more rapid transition of cells from G_0 to G_1 phase of the cell cycle (Allen et al., 1995). When quiescent satellite cells from the female *levator ani* muscle are exposed to testosterone, proliferation is induced as measured by uptake of ^{3}H-Thymidine (Joubert and Tobin, 1995). Yablonka-Reuveni et al. (1999), demonstrated that treatment of muscle fiber associated satellite cells with fibroblast growth factor-2 (FGF-2) induced satellite cells to enter the cell cycle as measured by expression of proliferating cell nuclear antigen (PCNA). Since satellite cells will remain quiescent when cultured in association with the muscle fiber (Bischoff, 1986a), these data demonstrate that satellite cell activity is in part regulated at the transition from quiescence.

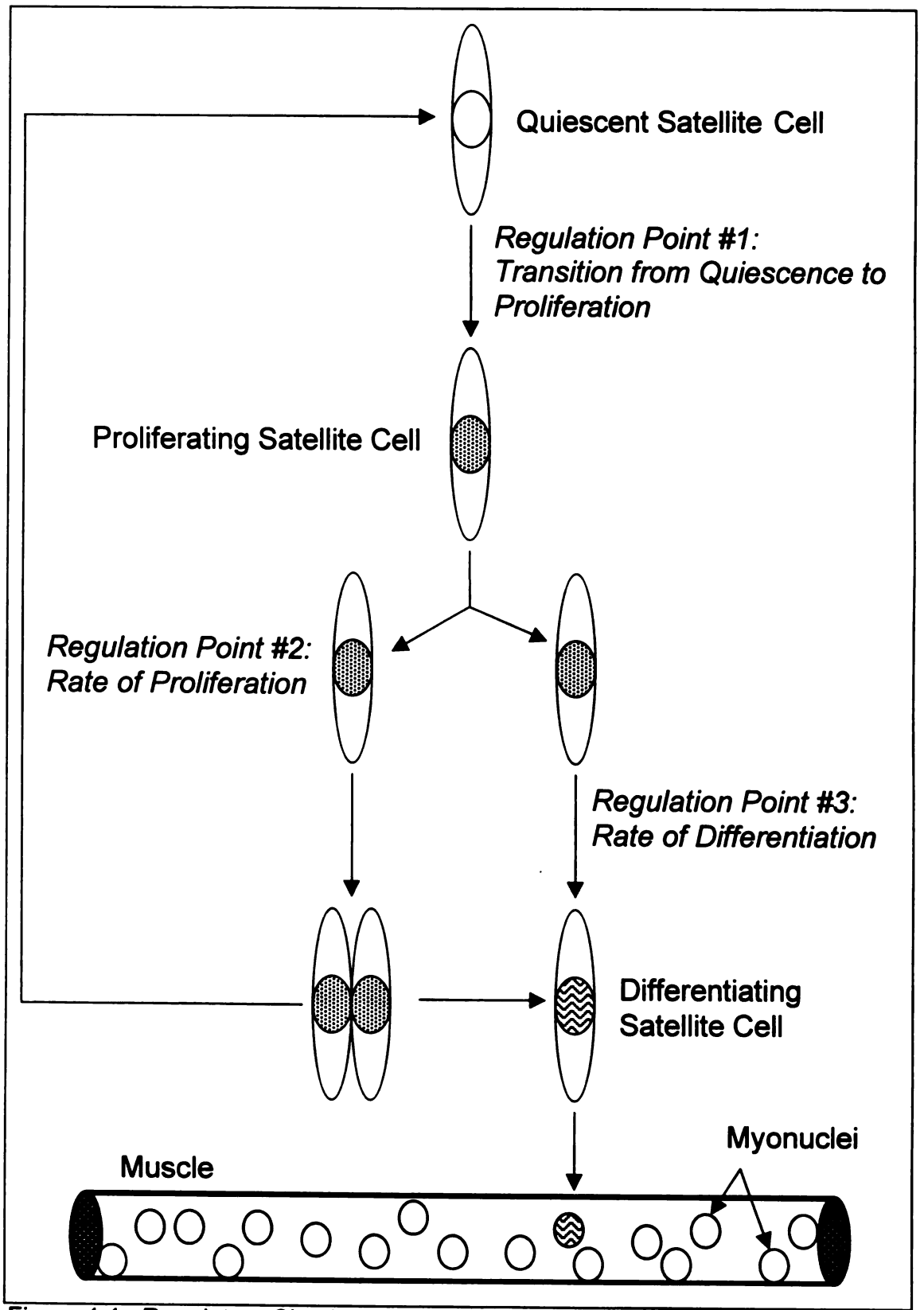


Figure 1.1. Regulatory Checkpoints in Satellite Cell Activity

The rate of proliferation is another checkpoint at which satellite cell activity is regulated (Figure 1.1.). The cell division cycle is made up of a 1) a pre-synthetic gap phase (G_1 phase), 2) a DNA synthesis phase (S phase), 3) a post-synthetic gap phase (G_2 phase), and 4) a mitosis phase (M phase). While S, M, and G_2 are relatively constant, the G_1 phase appears to be the most variable. Hence, the rate by which a satellite cell proliferates depends upon the time it takes to progress through G_1 (Allen, 1979). Turkey satellite cell clones isolated from the same animal have been shown to have different kinetics of proliferation under identical culture conditions (McFarland et al., 1995). Cell cycle time for satellite cells in 30-day-old growing rats has been estimated to be approximately 32 hours in length with an S phase of 14 hours (Schultz, 1996). In these rats, approximately 80% of satellite cells divide within a 32-hour cell cycle time as measured by 5-bromo-2'-deoxyuridine (BrdU) incorporation. The remaining 20% divide at a much slower rate, possibly entering a quiescent state before undergoing another division. Taken together, these data indicate that the rate of proliferation is a mechanism by which satellite cell activity may be modulated.

The rate at which satellite cells differentiate is another regulatory checkpoint for satellite cell activity (Figure 1.1.). Changes in the rate of differentiation affect the subsequent fusion of satellite cells with existing muscle fibers as well as the number of satellite cells that remain proliferating. Many growth factors and hormones may exert their effects in part through a decrease in differentiation (reviewed by Allen and Rankin, 1990; Dodson et al., 1996). Doumit et al. (1996) demonstrated that *in vitro* treatment of porcine satellite cell

clones with 10^{-7}M testosterone caused a decrease in differentiation as measured by a decreased percentage (relative to total nuclei) of myotube nuclei, nuclei expressing myosin heavy chain (MHC) protein, as well as a decrease in creatine kinase (CK) activity. These researchers postulated that a decline in satellite cell differentiation would result in an expanded population of proliferative satellite cells.

Age-related Changes in Satellite Cell Activity

Age-related changes in total satellite cell numbers and proliferative capacity have been reported previously. The percentage of total nuclei that were classified as satellite cells in the *subclavius* and *peroneus* muscles decreases from 30-35% in the neonate to about 5% in mature rats (Allbrook et al., 1971). Using colchicine to induce metaphase arrest, the incidence of mitotic figures in 10-week-old rats was roughly half of that in 3-week-old rats, indicating a reduced proliferative capacity as a function of age. These data are corroborated by studies of Cardasis and Cooper (1975), who reported a similar decline in the number of satellite cell nuclei in the *gastrocnemis* muscle of growing mice.

The *peroneus longus* and *sartorius* muscles of the pig also exhibit a decrease in the percentage of satellite cells as a function of age. The percentage of total nuclei classified as satellite cells decreased from approximately 15% in 1-week-old pigs to less than 5% in 64-week-old pigs (Campion et al., 1981). As in other studies, the percentage of total nuclei classified as myonuclei increased to 64 weeks of age, indicating DNA accretion

and hypertrophy of the muscles studied. However, these data suggest that absolute number of satellite cells may have increased from 1 to 32 weeks of age and was at least maintained from 1 to 64 weeks of age. Campion et al. (1982) reported that total numbers of satellite cells also increased from 4 days to 4 weeks of age in the *semimembranosus* muscle of both fast growing and normal Japanese quail. An increase in the total numbers of satellite cells has been observed in the *soleus* muscle of 1 to 12-month-old rats but not in *the extensor digitorum longus* muscle at similar ages (Gibson and Schultz, 1983). Collectively, it appears that a postnatal increase in absolute numbers of satellite cells is possible even if the proportion of satellite cells to total myonuclei declines throughout growth. Furthermore, the changes in the total number of satellite cells throughout growth depend in part on the muscles examined.

In vitro studies have also demonstrated age-related changes in satellite cell proliferation. When rat satellite cells are isolated and plated at clonal densities, the average number of cells per colony is inversely proportional to the age of the animal from which the cells were isolated (Schultz and Lipton, 1982). These data indicate that the proliferative capacity of satellite cells *in vitro* is dictated in part by the age of the animal. Similar differences are observed when satellite cells are plated in mass culture. Primary rat satellite cells isolated from 3-week-old rats have a shorter lag phase of PCNA expression *in vitro* when compared to cells isolated from 9-month-old rats (Johnson and Allen, 1993). Yablonka-Reuveni et al. (1999) demonstrated delayed expression of PCNA in muscle fiber-associated satellite cells isolated from 10-month-old rats when

compared to that of 3-week-old rats. These studies provide indirect evidence for an age-related decline in satellite cell proliferation.

Age-related differences have also been reported using muscle damage/regeneration models to study satellite cell activity. Six and 16-week-old rats were subjected to treadmill exercise to induce focal damage in the *soleus* muscle. 5-bromo-2'-deoxyuridine labeling index in sections of the *m. soleus* reached a maximum at 24 hours and 168 hours post-exercise in 6-week and 16-week-old rats, respectively (Jacobs et al., 1995). When rat primary satellite cell cultures are induced to proliferate by exposure to low-level irradiation, ^{3}H-Thymidine incorporation decreased with the age of rat from which the cells where isolated (Ben-Dov et al., 1999). These studies demonstrate a reduced capacity of cells undergoing proliferation to facilitate a regeneration response due to age.

The age-related changes in satellite cell activity are also influenced by the physiological status of the animal. Gonadally intact male pigs, castrated males, and castrated males implanted with testosterone propionate were sacrificed at 7, 14, and 21 days of age after a 6-hour continuous infusion of ^{3}H-Thymidine (Mulvaney et al., 1988). Myofiber segments were isolated from the *triceps brachii* muscle and total and labeled nuclei were enumerated. An age-related decline in satellite cell DNA synthesis was observed under each physiological condition. The rate of decline was greatest for castrated pigs, whereas the decline was attenuated in gonadally intact pigs and pigs treated with testosterone propionate. Clonally derived turkey satellite cells exhibit an age-related decline in proliferation when exposed to serum from hens at 3 to 15 weeks of age, but not

to serum from tom turkeys (Doumit et al., 1990). These data indicate that an age-related decrease in satellite cell activity may occur relatively early in postnatal life and may be modulated by hormonal status of the animal.

Post-hatch turkey skeletal muscles also exhibit age-related changes in satellite cell activity (Mozdziak et al., 1994). Tom turkeys at 3, 6, 9, 18, and 26 weeks of age were infused with BrdU to measure DNA synthesis. Myofiber segments with associated satellite cells were enzymatically isolated from the *pectoralis thoracicus* and *biceps femoris* muscles and stained for the presence of BrdU. Myofiber diameter and cytoplasmic volume to nucleus ratio (CNR) were also measured. Myofiber diameter increased throughout the entire experimental period, indicating muscle hypertrophy. From 3 to 6 weeks of age, CNR increased in association with a high degree of DNA synthesis, expressed as the number of BrdU labeled nuclei per 1000 total nuclei. Between 6 and 9 weeks of age, no changes occurred in CNR, but DNA synthesis declined to low levels. At later stages of growth (9-26 weeks of age), CNR increased steadily whereas DNA synthesis continued to decline (Mozdziak et al., 1994). These data indicate that DNA synthesis is important relatively early in turkey skeletal muscle growth and that increases in CNR constitute the majority of the hypertrophy response in the later stages of growth. When satellite cell DNA synthesis is abolished through irradiation in the left *pectoralis thoracicus* muscle of 3-week-old turkeys, muscle weight is depressed up to 15 weeks after treatment when compared to contra-lateral control (Mozdziak et al., 1997). Irradiation also caused a depression in the number of nuclei per myofiber length due to a decrease in DNA synthesis at

1 and 4 weeks after irradiation. Since CNR is not different at any time after treatment, these data demonstrate that inhibition of high levels of DNA synthesis at 3-6 weeks of age depresses subsequent myonuclear accretion and muscle growth.

Relatively few data exist on the effects of age on satellite cell differentiation and fusion. A study by Allen et al. (1982) examined the effect of donor age on the ability of satellite cell-derived myotubes to accumulate muscle-specific proteins *in vitro*. Satellite cells were isolated and cultured from neonatal rats (<5 days of age), growing rats (1-3 months of age), adult rats (9-12 months of age) and old rats (>24 months of age), allowed to differentiate and incorporate into myotubes *in vitro*, then assayed for accumulation of α-actin protein. Cultures from neonatal rats were shown to accumulate significantly more α-actin than growing, adult or old rats, but no significant differences were observed between these later ages. Although this study did not measure differentiation directly, it appears that muscle-specific protein accumulation does not vary during the later stages of postnatal growth.

In vitro measures also have been used to determine if age-related changes in satellite cell differentiation exist. Satellite cells were isolated from the *pectoralis major* muscle of 3, 9, and 15-week-old turkeys, grown to near-confluence in culture, trypsinized and passaged. Tertiary culture satellite cells derived from 9 and 15-week-old turkeys appeared to have a greater capacity to form myotubes *in vitro* as measured by the percentage of total nuclei in myotubes (fusion percentage) (Doumit et al., 1990). Although it is possible that

satellite cells may have lost an *in vivo* phenotype when cultured, these results indicate that satellite cells from older animals retain the capacity to differentiate and fuse *in vitro*.

Although a number of studies demonstrate that indices of satellite cell activity decline as a function of age, many of these studies used non-meat animal species. Due to animal expenses as well as the prohibitive costs of radioactive isotopes and animal disposal, studies examining satellite cell activity in species used for meat production have been confined to the earliest stages of postnatal growth. Therefore, characterization of satellite cell activity throughout postnatal growth for meat animal species is required so that strategies can be developed and employed to optimize satellite cell activity which will in turn impact DNA accretion and ultimately muscle growth.

Age-related changes in satellite cell activity may be explained in part by changes in the intercellular concentrations of growth factors. Stimulation of satellite cell proliferation by homogenized muscle extracts was inversely proportional to the age of rat from which the muscles were isolated (Mezzogiorno et al., 1993). Furthermore, conditioned media from myotube cultures derived from young rats stimulated myosin heavy chain (MHC) expression in satellite cell cultures from 1 and 26-month-old rats. However, conditioned media from myotube cultures of old mice failed to stimulate MHC expression in satellite cells from either young or old animals. These data demonstrate that age-related changes in satellite cell activity may be modulated in part by the paracrine secretion of growth factors.

Effects of Growth Factors on Satellite Cell Activity

A. Hepatocyte Growth Factor (HGF)

In classical studies by Bischoff (1986b), crushed muscle extracts (CME) were demonstrated to contain mitogenic factors that induce quiescent satellite cells to enter the proliferative state. When cultures of single viable muscle fibers from rat *flexor digitorum brevis* muscle were exposed to CME in basal medium, quiescent satellite cells were induced to enter the cell cycle *in vitro*. Specificity was confined to myogenic cells, as fibroblast cultures treated with CME did not accumulate significant amounts of ^{3}H-Thymidine. Direct injections of CME and ^{3}H-Thymidine into the *flexor digitorum brevis* muscle of 1-week-old rat pups demonstrated that satellite cell proliferation can be stimulated *in vivo*, causing increases in total DNA content and myonuclei per fiber (Bischoff, 1986b).

Attempts to characterize the unknown mitogen in CME revealed that it had a molecular mass greater than 30 kilodaltons and was heat and trypsin sensitive (Bischoff, 1986b). In an effort to further characterize the mitogen, Chen et al. (1992) introduced CME with saturating concentrations of FGF-2, insulin-like growth factor-I (IGF-I), transferrin, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), adrenocorticotropic hormone (ACTH) and macrophage colony-stimulating factor (M-CSF) to myogenic cultures isolated from 5-day-old Swiss-Webster mice. Crushed muscle extract acted additively with these growth factors to promote proliferation, indicating that the unknown factor responsible for the mitogenicity of CME is distinct from those investigated

(Chen et al., 1992). In an attempt to further identify the factor(s) responsible for its activity, CME was then separated into distinct mitogenic activities using heparin affinity chromatography (Chen et al., 1994). Crushed muscle extract was found to contain a protein that shares antigenicity with the BB isoform of PDGF, as well as another protein that elutes from the column under identical conditions as FGF-2. In addition, an unknown mitogenic component eluted from the column at 0.9 M NaCl. This mitogen was shown to act additively with a combination of saturating doses of FGF-2, IGF-I and EGF and also preferentially stimulated proliferation in C2 myoblasts but not fibroblasts (Chen et al., 1994). More recently, hepatocyte growth factor (HGF) was identified as the unknown component of CME using western immunoblotting (Tatsumi et al., 1998). Furthermore, pre-incubation of CME with anti-HGF antibodies abolished the mitogenic activity of CME.

Hepatocyte growth factor can also increase the rate of proliferation of those satellite cells that have entered a proliferative state. Satellite cells isolated and cultured from 9-month-old Sprague Dawley rats were shown to have a lag phase leading to division of approximately 42 to 60 hrs. However, in the presence of 10 ng/ml HGF, cells enter the cell cycle earlier and show a significant increase in cell number as early as 24 hours in culture when compared to control cultures. This increase in cell number is accompanied by accelerated expression of cyclin D1 and PCNA, proteins required for progression through the cell cycle. Messenger RNA for c-met, the HGF receptor, has been detected in satellite cells from 0 to 72 hours in culture (Allen et al., 1995).

Injection of HGF into the *tibialis anterior* muscle of 12-month-old rats causes quiescent satellite cells to enter the cell cycle and initiate DNA synthesis as measured by BrdU incorporation (Tatsumi et al., 1998). Hepatocyte Growth Factor at 20 ng/ml has also been shown to increase the rate of DNA synthesis of proliferating chicken satellite cells (Gal-Levi et al., 1998).

Hepatocyte growth factor also depresses differentiation of satellite cells. Ectopic expression of chicken HGF by chicken satellite cells depressed expression of MyoD and myogenin mRNA (Gal-Levi et al., 1998). MyoD and Myogenin are muscle-specific transcription factors expressed early in the differentiation program (Smith et al., 1994; Cornelison and Wold, 1997). Furthermore, ectopic expression of HGF suppressed expression of MHC protein in these cells (Gal-Levi et al., 1998). Further studies indicated that depression of transcription factor expression and differentiation may be a *c-met* mediated event. When C2 mouse myoblasts are transfected with a constitutively activated catalytic domain of *c-met*, expression of MyoD and myogenin mRNA as well as MHC protein is depressed, indicating a depression of differentiation (Anastasi et al., 1997). Since *c-met* mRNA is down-regulated preceding differentiation of wild-type C2 myoblasts (Gal-Levi et al., 1998), it appears that differentiation may occur through down-regulation of the *c-met* receptor.

Hepatocyte growth factor and its receptor *c-met* have been implicated in modulating satellite cell activity in an autocrine action. Hepatocyte growth factor is localized to the extracellular matrix surrounding rat muscle fibers, and the associated satellite cells express *c-met* (Tatsumi et al., 1998). In regenerating

muscle, HGF appears to be released from the extracellular matrix and is colocalized with immunostaining for *c-met* on the surface of satellite cells. Hepatocyte growth factor mRNA has been detected in myotubes derived from chicken satellite cells and C2 myoblasts (Gal-Levi et al., 1998) as well as in proliferating C2 myoblasts (Anastasi et al., 1997). C2 cultures exposed to an acid treatment followed by 0.5 M NaCl to remove HGF from the extracellular matrix and dissociate receptor-ligand complexes results in a net decrease in *c-met* tyrosine phosphorylation. Application of C2 conditioned media to these cultures restores phosphorylation of the receptor, indicating that HGF interacts with the *c-met* receptor in an autocrine fashion (Anastasi et al., 1997).

Hepatocyte growth factor secretion by muscle appears to be developmentally regulated. HGF mRNA has been detected in fractionated muscle RNA isolated from 2, 4 and 10-day-old rats, but cannot be detected in muscle RNA isolated from 35-day-old rats. However, HGF mRNA is reexpressed in regenerating muscle and satellite cells of adult rats 3 to 4 days after ischemic injury (Jennische et al., 1993). It is unknown if the developmental decline in muscle HGF may be in part responsible for the observed age-related transition of proliferating satellite cells to the quiescent state.

B. Fibroblast Growth Factor-2 (FGF-2)

Fibroblast growth factor-2 has also been implicated in activating quiescent satellite cells to enter a proliferative state. Cultures of rat muscle fibers treated with FGF-2 or HGF exhibit increases in the number of PCNA+ satellite cells by

day 2 *in vitro*. However, PCNA labeling in HGF treated fiber cultures decreased at day 4 in culture, yet FGF-2 treated cultures did not exhibit a decrease until day 5. These data indicated that FGF-2, in addition to activating quiescent satellite, is also capable of maintaining cells in a proliferative state (Yablonka-Reuveni et al., 1999).

Early studies by Gospodarowicz et al. (1976) demonstrated that the FGF family of growth factors was capable of influencing myoblast proliferation and differentiation. Using myoblasts isolated from bovine fetuses, FGFs purified from either bovine brain or pituitary gland stimulated proliferation *in vitro*. Furthermore, cultures maintained in the presence of FGF did not differentiate and fuse to form multinucleated myotubes to the same extent as control cultures. Both pituitary and brain derived FGFs have been reported to repress muscle CK activity in the BC_3H1 clonal mouse muscle cell line, indicating a depression of differentiation and muscle-specific gene expression (Lathrop et al., 1985). However, pituitary-derived FGF also caused an increase in proliferation in the BC_3H1 cell line, whereas brain FGF did not elicit a response. These data demonstrate that FGFs are capable of stimulating proliferation and depressing diffferentiation in myogenic cells.

Fibroblast growth factor-2 has been immunolocalized to the heparin-containing basal lamina of mouse skeletal muscle fibers (DiMario et al., 1989), yet conflicting data exist on the cellular origins of secreted FGFs. Rat primary satellite cells have been shown to express acidic-FGF (FGF-1) protein, but not basic-FGF (FGF-2) (Groux-Muscatelli et al., 1990). However, FGF-2 appears to

be approximately 30-fold more potent than FGF-1 in stimulating proliferation of the MM14 mouse myoblast cell line (Clegg et al., 1987). Both FGF-1 and FGF-2 mRNA have been detected in proliferating myoblasts but are absent from differentiating cells of the MM14 cell line (Hannon et al., 1996). Furthermore, transfection of these cells with FGF-1 and FGF–2 expression constructs mimics the effects of exogenously applied FGFs, indicating that these cells are capable of secreting functional FGFs. Although the origins of FGF-2 *in vivo* remain unresolved, FGF-2 is a potent mitogen for satellite cell mediated skeletal muscle growth.

Fibroblast growth factor-2 appears to act through maintenance of a proliferative state, thereby depressing differentiation and muscle-specific gene expression. Growing MM14 myoblasts become postmitotic, begin to express muscle-specific genes, then fuse within 12-14 hours after FGF-2 withdrawal (Clegg et al., 1987). Commitment to differentiation occurs in the G_1 phase of the cell cycle, with those cells in a post-G_1 state completing the cell cycle before commitment to terminal differentiation. Serum and FGF-2 are required to maintain proliferation in this cell type. In the absence of serum, cultures can be maintained in a post-mitotic state without commitment to differentiation. These data indicate that repression of differentiation and stimulation of proliferation by FGF-2 occurs through independent.

Developmental changes in FGF-2 levels as well as changes in the mitogenic effect of FGF-2 on satellite cells have been investigated. FGF-2 mRNA levels in skeletal muscle are initially high at 90 days of gestation in the pig

fetus, but decline until birth and remain constant to 180 days after birth (Peng et al., 1997). Satellite cells isolated from 3-4-week-old rats exhibit specific, high affinity binding of FGF2 to the fibroblast growth factor receptor-1 (FGFR-1) *in vitro* as early as 18 hours post-plating, whereas cells from 9-12-month-old rats do not exhibit binding until 42 hours post-plating (Johnson and Allen, 1993). These data may in part explain the extended lag phase observed when satellite cells are isolated and cultured from older rats. Yablonka-Reuveni et al., (1999) demonstrated an increase in both the number of satellite cells (mitogen-activated protein kinase positive cells (MAPK+)) as well as a number of proliferating satellite cells (MAPK+/PCNA+) due to FGF-2 treatment of muscle fiber cultures isolated from 3-week, 8-week and 10-month-old rats. Interestingly, The number of MAPK+ and MAPK+/PCNA+ satellite cells were higher for 10-month-old rats when compared to the younger rats. Furthermore, treatment of cultures with FGF-2 caused a greater number of satellite cells to enter the myogenin+ compartment, indicating that differentiation was enhanced in this culture system. It is possible that these conflicting results are a result of the continued association of satellite cells with the native fiber, or secretion of growth factor(s) by the fibers themselves. It is unknown if FGF-2 is responsible for regulating age-related changes in satellite cell activity through activation of quiescent satellite cells. Furthermore, the role of FGF-2 in maintaining the proliferative state throughout growth remains to be elucidated.

C. Insulin-like Growth Factor-I (IGF-I)

Insulin-like growth factor-I is unique among growth factors because it is able to elicit both mitogenic and myogenic responses, through interaction with the same receptor (Insulin-like growth factor receptor-1 (IGFR-1)) (Florini et al., 1996). Insulin-like growth factor-I increased proliferation of L6 myoblast cultures and increased expression of *c-fos* mRNA and Cyclin D protein (Coolican et al., 1997). In cultures of differentiating L6 myoblasts, IGF-I also increased CK activity (Florini et al., 1997; Coolican et al., 1997), and up-regulated expression of myogenin mRNA and protein (Florini et al., 1997). It appears that stimulation of proliferation and differentiation occurs through activation of MAPK kinase and phosphatidylinositol 3-kinase/$p70^{S6k}$ pathways, respectively (Coolican et al., 1997).

Interestingly, IGF-I appears to have synergistic effects on satellite cell proliferation and differentiation when in the presence of FGF-2. When rat satellite cells are exposed to a combination of FGF-2 and IGF-I, proliferation and differentiation are synergistically enhanced (Allen and Boxhorn, 1989). When porcine myogenic clones were treated with FGF-2 and IGF-I, a synergistic stimulation of proliferation was observed (Doumit et al., 1993). The mechanism for this synergistic response is unknown. The potential interactions between IGF-I and HGF on porcine satellite cell activity also remain to be established.

Insulin-like growth factor binding proteins (IGFBPs) are also capable of modulating the response of satellite cells to IGF-I. Insulin-like growth factor binding protein-4 is capable of depressing both proliferation and differentiation of

the L6 myoblast cell line. However, IGFBP-5 inhibits proliferation but enhances differentiation of L6 myoblasts. It appears that the role of IGFBP-4 is mainly to sequester excess IGFs, whereas expression of IGFBP-5 elicits a dual response, possibly due to its ability to associate with the cell membrane (Ewton et al., 1998).

The IGF-I regulatory system appears to be regulated in skeletal muscle throughout growth. Muscle IGF-I mRNA levels are reported to be low at 30 days of gestation in the pig fetus, increase between 44 and 59 days and plateau until 75 days, where levels rise again and peak at birth (Gerrard et al., 1997). In agreement with these findings, Peng et al. (1996) reported that IGF-I mRNA levels are initially high in pig fetuses from 90 days to 110 days of gestation, then decline from birth to 180 days of age. Similarly, IGF receptor-1 (IGFR-1) mRNA is also reported to be high at 110 days of gestation, then declines to basal levels at 21 days after birth and remain constant throughout postnatal life. IGF binding protein-3 (IGFBP-3) mRNA levels decline from high levels at 90 days of gestation to basal levels at 21 days after birth and remain low to 180 days (Peng et al., 1996). Insulin-like growth factor-I mRNA and protein have both been localized to the developing muscle fibers (Gerrard et al., 1997). Taken together, these data demonstrate that levels of muscle IGF-I, IGFR-1 and IGFBP-3 transcripts are high in the pig fetus before birth, then decline throughout postnatal development. The relationship between satellite cell activity and the developmental changes in IGFI, IGFR-1 and IGFBPs are currently unresolved.

Several lines of evidence indicate that age-related changes in satellite cell proliferation and differentiation are mediated through changes in the intercellular levels of specific growth factors. In order to develop strategies to optimize skeletal muscle growth in meat animal species, the mechanisms by which these growth factors affect satellite cell activity need to be elucidated. Of the possible candidates, HGF, FGF-2 and IGF are most likely the major mediators of skeletal muscle DNA accretion. Collectively, these growth factors have the ability to activate quiescent satellite cells, as well as stimulate proliferation and/or differentiation. Interactions among these growth factors need to be elucidated. Additionally, tissue levels of these growth factors and their influence on DNA accretion and skeletal muscle growth *in vivo* remain to be established.

Based on the evidence presented above, I hypothesized that populations of proliferating and differentiating satellite cells decline *in vivo* from 1 to 21 weeks of age and that this decline limits DNA accretion and subsequent skeletal muscle growth. The following objectives were designed to test this hypothesis:

Objective 1: Develop an immunocytochemical staining system to quantify *in vivo* populations of proliferating and differentiating satellite cells.

Objective 2: Characterize the age-related changes in satellite cell proliferation and differentiation from 1 to 21 weeks of age.

Objective 3: Relate changes in satellite cell activity to DNA accretion and postnatal skeletal muscle growth.

CHAPTER 2

QUANTIFICATION OF SATELLITE CELL PROLIFERATION AND DIFFERENTIATION IN GROWING PIGS FROM 1 TO 21 WEEKS OF AGE

Abstract

Postnatal muscle DNA accumulation occurs through proliferation of satellite cells followed by differentiation and fusion of these cells with existing muscle fibers. Furthermore, muscle DNA accumulation is proportional to fiber diameter or growth. Therefore, characterization of the age-related changes in satellite cell activity is essential to the understanding of skeletal muscle growth. Satellite cells were isolated from the *semitendinosus* (ST) muscle of pigs at 1, 7, 14, and 21 weeks of age (4 animals/age group). Satellite cells were separated from cellular debris using Percoll gradient centrifugation, and adsorbed to glass coverslips for fluorescent immunostaining. Positive staining for neural cell adhesion molecule (NCAM) distinguished satellite cells from non-myogenic cells. Proliferating cell nuclear antigen (PCNA) and myogenin were used as markers for quantification of proliferating and differentiating satellite cells, respectively. DNA and protein concentrations of the ST and *longissimus dorsi* (LD) were determined and used to calculate total protein and DNA accretion as well as the protein:DNA ratio. Accretion of DNA and protein closely paralleled increases in muscle weights of the ST and LD. Furthermore, the protein to DNA ratio remained constant in the ST and LD from 1 to 7 weeks of age, then increased for both muscles from 7 to 21 weeks of age. The proportion of satellite cells (NCAM+ cells) was highest in

isolates from 1-week-old pigs, yet the proportion of proliferating satellite cells (NCAM+/PCNA+ cells) declined only slightly with age. Furthermore, a high degree of proliferation (>75% PCNA positive nuclei) was maintained to 21 weeks of age. The percentage of differentiating satellite cells decreased from 30% at 1 week to 14% at 7 weeks of age and remained at constant levels thereafter. These data indicate that a high percentage of satellite cells remain proliferative throughout postnatal growth in pigs from 1 to 21 week of age. Differentiation may be a critical step in the regulation of postnatal skeletal muscle hypertrophy.

Introduction

Postnatal skeletal muscle growth in the pig occurs through hypertrophy of existing muscle fibers, since the number of muscle fibers is determined prenatally and remains constant throughout postnatal life (Stickland et al., 1975; Wigmore and Stickland, 1982, 1983). Muscle fiber hypertrophy is associated with an increased DNA content, yet myonuclei do not retain the ability to synthesize DNA (Stockdale and Holtzer, 1961). Satellite cells, first discovered by Alexander Mauro in 1961, are the sole postnatal source of DNA contributed to growing muscle fibers. DNA accretion occurs through proliferation of satellite cells followed by differentiation and fusion with existing muscle fibers (Moss and LeBlond, 1971).

It is generally accepted that the proportion of satellite cells and proliferating satellite cells declines with age. Campion et al. (1981) observed a 10% decrease in the proportion of nuclei classified as satellite cells in histological

sections of the *peroneus longus* and *sartorius* muscles from 1 to 64-week-old pigs. However, these data suggest that the absolute number of satellite cells in these muscles may actually increase from 1 to 32 weeks of age. Increases in the absolute number of satellite cells throughout postnatal growth have also been reported for the *semimembranosus* muscle of Japanese quail (Campion et al., 1982) and the *soleus* muscle of the rat (Gibson and Schultz, 1983). Mulvaney et al. (1988) demonstrated a decrease in satellite cell proliferation (based on total myonuclei) as measured by incorporation of ^{3}H-thymidine into satellite cells of the *triceps brachii* muscle of growing pigs 1 to 21 days of age. Other investigators have demonstrated similar declines in both the proportion of satellite cells as well as the percentage of proliferating satellite cells in the later stages of growth in laboratory animals (Allbrook et al., 1971; Cardasis and Cooper, 1975; Schultz and Lipton, 1982). The age-related changes in satellite cell proliferation throughout the rapid growth phase of porcine skeletal muscle have not been characterized.

The rate of differentiation may be a potentially important modulator of muscle growth as it regulates both the accretion of DNA into muscle fibers as well as the number of satellite cells that remain capable of proliferation. Quinn et al. (1990) demonstrated that embryonic myoblasts isolated from fetal calves with the double-muscling phenotype exhibited a delay in differentiation compared to myoblasts isolated from normal fetuses. This delay resulted in an enlarged population of myoblasts resulting in an increased production of fused myotubes *in vitro*. Coutinho et al. (1993) observed a similar delay in formation of the

brachial somites in quail embryos selected for rapid muscle growth. These data indicate that the rate of differentiation may be a critical event in muscle development. To date, the age-related changes in satellite cell differentiation during postnatal skeletal muscle growth have not been characterized.

Characterization of the age-related changes in satellite cell proliferation and differentiation will provide insight into the cellular mechanisms governing skeletal muscle hypertrophy. This information will be useful for development of strategies to increase DNA accretion and skeletal muscle growth. Such advances will also serve to increase the efficiency of lean tissue accretion throughout postnatal growth.

Materials and Methods

A. Animals

Duroc sires were mated to F_1 Yorkshire x Landrace sows and F_2 progeny were raised at the Michigan State University Swine Farm. Male piglets were castrated at 1 day of age. F_2 barrows and gilts at 1, 7, 14, and 21 weeks of age (4 animals per age group) were transported to the Michigan State University Meat Laboratory and slaughtered following procedures outlined in the Code of Federal Regulations for Humane Slaughter of Livestock (Sections 313.1-313.9).

B. Satellite Cell Isolation

Satellite cells were isolated from the right hindlimb muscles of growing pigs as described by Doumit and Merkel (1992). Due to low muscle mass of 1-

week-old pigs, satellite cells were isolated from both the *semimembranosus* and *semitendinosus* muscles, while only the *semitendinosus* was used from 7, 14, and 21-week-old pigs. Briefly, muscles were removed from the right hindlimb, trimmed, weighed and rinsed in cold phosphate buffered saline pH=7.4 (PBS). In a laminar flow tissue culture hood, muscle was cut into ~2 cm^3 sections and visible connective tissue was removed. Sections were then washed in cold Minimum Essential Medium (MEM) pH=7.1 (11900-024, Gibco BRL, Grand Island NY) and ground in an aseptically prepared meat grinder. Thirty grams of muscle tissue were digested in PBS containing 0.8 mg/ml Pronase E (P-8811, lot#88H1351, 3.9 units/mg, Sigma Chemical Co., St Louis MO) for 50 minutes at 37°C. Samples were vortexed at 10-minute intervals during enzymatic digestion. Digestions were performed at a ratio of 40% tissue to 60% protease solution (w/v). After digestion, samples were separated from the protease solution by centrifugation at 1000 *xg* followed by washing of the pellets with PBS. Satellite cells were separated from debris by differential centrifugation at 100 *xg*. Satellite cells were collected by centrifugation at 1000 *xg* and pellets were re-suspended in MEM supplemented with 20% Fetal Bovine Serum (FBS)(F-2442, Lot#76H4647, Sigma Chemical Co., St Louis MO), 0.5% Antibiotic/Antimycotic and 0.1% Gentamicin (A-5955 and G-1397, Sigma Chemical Co., St Louis MO). Cell suspensions were filtered through ~500 μm and double layered ~53 μm Nitex cloth (3-500-49 and 3-53-41, Sefar America, Kansas City MO). Dimethyl sulfoxide (#9224-01, J.T. Baker, Phillipsburg NJ) was added to the cell

suspension to 10% of the total volume and aliquots were frozen overnight in styrofoam containers at -80°C and stored in liquid nitrogen until use.

C. Tissue Collection

Samples were taken from the left *semitendinosus* (ST) and right *longissimus dorsi* (LD) muscles for determination of DNA and protein concentrations. The ST was excised completely, trimmed of connective tissue, weighed and samples were collected from the belly of the muscle. For 1 and 7-week-old pigs, the LD was removed quantitatively from the cranial end of the ilium to its termination in the shoulder then weighed. *Longissmus dorsi* samples were taken from the region of the muscle adjacent to the 10^{th} rib. In 14 and 21-week-old pigs, samples were prepared from a 25-100mm thick section that was excised immediately caudal to the 10^{th} rib. The remainder of the LD was removed quantitatively as described for 1 and 7-week-old pigs to determine total muscle weight.

For histology studies, 5x5x10 mm samples were excised with the long axis of the sample running parallel to the muscle fibers. Segments were coated in Tissue-Tek O.C.T. Compound (4583, Sakura Finetek U.S.A. Torrance CA) then frozen in 2-methylbutane (#32,040-4, Aldrich Chemical Company, Milwaukee, WI) that had been cooled in liquid nitrogen vapors. The remainder of the muscle section was cut into 1-2 cm^3 sections, frozen in liquid nitrogen, and stored at -80°C.

At exsanguination, 100 ml of blood was collected for serum isolation. Blood was allowed to clot at room temperature for 2 hours, then stored at 4°C overnight. Blood was centrifuged at 2000 *xg* for 30 minutes at 4°C and serum was collected. Serum was centrifuged again, transferred to a new tube and frozen at -80°C.

Representative samples of liver were also taken for further analysis. Liver samples were cut into 1-2 cm^3 sections, frozen in liquid nitrogen and stored at -80°C.

D. DNA Assay

The DNA contents of the left *semitendinosus* and right *longissimus dorsi* muscles were determined using the procedure of Labarca and Paigen (1980). Briefly, 1.0 g of muscle was homogenized (4, 30-second bursts) in 25 volumes (w/w) of cold extraction buffer (10 mM Tris, 5 mM EDTA, pH=8.0) using a Polytron homogenizer (Brinkmann, Westbury NY). Triplicate 100 µl aliquots of muscle homogenate were mixed with 3 ml of DNA assay buffer (0.05 M $NaH_2PO_4 \bullet H_20$, 2 M NaCl, 2 mM EDTA, pH=7.4 containing 1 µg/ml Hoechst 33258 reagent (B-2883, Sigma Chemical Co., St. Louis MO) in methacrylate cuvets (14-38621, Fisher Scientific, Itasca IL) pre-selected for optical uniformity. A serial dilution (100 to 3.125 µg/ml) of Calf Thymus DNA (D-0805, Sigma Chemical Co., St. Louis MO) in extraction buffer served as a standard and was treated in an identical fashion to the samples. Samples and standards were

incubated in the dark for 15 minutes then read with a DynaQuant fluorometer (Hoefer Pharmacia Biotechnology Inc., San Francisco CA).

E. Biuret Protein Assay

Determination of protein content of the left *semitendinosus* and right *longissimus dorsi* muscles was accomplished using aliquots of the muscle homogenate following a procedure outlined Gornall et al. (1948) and modified by Robson et al. (1968). Briefly, triplicate 250 µl aliquots were treated with 750 µl of 1 N NaOH, vortexed and incubated at 37°C for 3-4 hours. A serial dilution of bovine serum albumin (A-2153, Sigma Chemical Co., St. Louis MO) in 25% extraction buffer, 75% 1 N NaOH served as a standard and was treated in an identical fashion to the samples. Four milliliters of biuret reagent (6 mM $CuSO_4 \bullet 5H_20$, 20 mM $KNaC_4H_4O_6 \bullet 4H_2O$, 750 mM NaOH) were added to samples and standards and incubated in the dark for 30 minutes. Two hundred and fifty microliters of sample and standards were placed into an Immulon 1 96 well microtiter plate (#14 24578, Fisher Scientific, Itasca IL) and read at 540nm.

F. Culture of 5.1H11 and F5D Hybridoma Cell Lines

The 5.1H11 cell line was developed by Drs. Helen Blau and Frank Walsh at Stanford University School of Medicine, Stanford CA. The F5D cell line was developed by Dr. Woodring Wright at the University of Texas Southwestern Medical Center, Dallas TX. The cell lines were obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa, Department of

Biological Sciences, Iowa City, IA 52242 under contract NO1-HD-7-3263 from the National Institute of Child Health and Human Development (NICHD).

5.1H11 hybridoma cells were grown in 75 cm^2 tissue culture flasks (#3376, Corning Incorporated, Corning NY) in Dulbecco's Modified Eagle Medium with 25 mM HEPES (#23700-040, Gibco BRL, Grand Island NY) containing 10% FBS, 0.1 mM Non-Essential Amino Acids (#11140-050, Gibco BRL, Grand Island NY), 1 mM sodium pyruvate (S-8636, Sigma Chemical Co., St. Louis MO), 2 mM L-glutamine (G-5763, Sigma Chemical Co., St. Louis MO) and 0.5% Antibiotic/Antimycotic. F5D hybridoma cells were grown in 75 cm^2 tissue culture flasks in RPMI medium 1640 (#31800-022, Gibco BRL, Grand Island NY) containing 10% FBS and 0.1% Gentamicin. All cultures were maintained in a 37°C humidified incubator containing 95% air and 5% CO_2. Frozen cell suspensions were thawed in a warm water bath and re-suspended in 10 ml of media to dilute freezing medium and centrifuged at 300 *xg* for 2 minutes. The cell pellet was then re-suspended and plated at $2.5x10^6$ cells/75 cm^2 flask in 20 ml of medium (125,000 cells/ml). Cells were supplied with new medium every 48 hours at a ratio of 1:4 (existing cell suspension to fresh media) and cell suspensions were transferred into new flasks when necessary. Feeding was continued until the desired volume of supernatant was attained. Cultures were then left undisturbed for 12-15 days. Cultures were harvested, pooled and centrifuged at 2000 *xg* for 15 minutes to pellet cells. The clarified supernatant was then collected and stored in aliquots at -20°C until use. Thawed aliquots were stored at 4°C.

G. Satellite Cell Enrichment and Adsorption to Coverslips

Porcine satellite cells were separated from debris using Percoll gradient centrifugation as described by Yablonka-Reuveni (1989). Briefly, a 90% Percoll solution (P-1644, Sigma Chemical Co., St Louis MO) was prepared in 10x MEM. Percoll solutions (60% and 20%) were prepared in 1x MEM as dilutions of the 90% stock. Two milliliters of 60% Percoll was added to a 15 ml Corex tube that had been pre-treated for 2 hours with either FBS or horse serum (H-1270, Sigma Chemical Co., St. Louis MO). Eight milliliters of 20% Percoll solution was then layered upon the 60% solution in the tube. One vial of primary muscle cell suspension was thawed in a warm water bath and transferred to a 15 ml conical tube. Two milliliters of MEM were used to wash the vial(s) and was added to the cell suspension. Diluted cell suspensions were loaded onto the gradients and centrifuged at 15,000 *xg* for 5 minutes at 4°C with the brakes off. The interface between the 20% and 60% Percoll solutions was recovered and diluted with 20 volumes of MEM. The diluted interface was then centrifuged at 300 *xg* for 15 minutes to pellet cells. The supernatant was then decanted and the cell pellet was re-suspended in the remaining volume (0.5-1 ml) of MEM. The cell suspension was transferred to a microcentrifuge tube and centrifuged at 1400 *xg* for 5 minutes. The supernatant was aspirated and the cell pellet was re-suspended in 200 µl of MEM. One hundred microliters of the resulting cell suspension was allowed to adsorb to 12 mm glass coverslips (12-545-82, Fisher Scientific, Itasca IL) for 45 minutes. For each satellite cell isolate, cells were

adsorbed onto two coverslips for staining for PCNA and Myogenin, respectively. Coverslips with adsorbed cells were washed once with PBS+2% Goat Serum (G-6767, Sigma Chemical Co., St Louis MO) before blocking and staining.

H. Immunostaining for Neural Cell Adhesion Molecule (NCAM)

All washes and antibody incubations were performed in a volume of 0.5 ml. Porcine satellite cells were incubated in PBS with 2% goat serum (blocking solution) for 5-10 minutes to block non-specific binding of antibodies. Cells were then incubated in undiluted 5.1H11 hybridoma supernatant (containing anti-NCAM antibodies) for 30 minutes (see Table 2.1. for description of antigen), followed by a series of three washes in blocking solution. Incubation of 1 μg/ml non-specific mouse IgG (I-5381, Sigma Chemical Co, St Louis MO) in the place of 5.1H11 hybridoma supernatant served as a negative control and subsequent steps were performed in an identical fashion. Cells were incubated for 30 minutes in blocking solution with 0.4 μg/ml biotinylated goat, anti-mouse IgG1 (#M32015, Caltag Laboratories, Burlingame CA), and the washing series was repeated. To detect primary antibody binding, cells were incubated for 30 minutes in the dark with ExtrAvidin conjugated to tetramethylrhodamine isothiocyanate (TRITC) (E-3011, Sigma Chemical Co., St Louis MO) diluted 1:200 in blocking solution. Cells were washed once in blocking solution then fixed with 1% formalin in PBS (#2106-01, J.T. Baker, Phillipsburg NJ) for 10 minutes, followed by a 10-minute exposure to -20°C methanol (#9070-03, J.T.

Baker, Phillipsburg NJ). The washing series was repeated and cells were stained for either the presence of PCNA or Myogenin.

I. Immunostaining for Proliferating Cell Nuclear Antigen (PCNA)

Porcine satellite cells that had been stained for the presence of NCAM (described above) were incubated in 1 μg/ml anti-PCNA monoclonal antibody (#1486 772, Boehringer Mannheim, Indianapolis IN) in blocking solution for 1-2 hours (see Table 2.1 for description of antigen). Incubation of 1 μg/ml non-specific mouse IgG in the place of anti-PCNA antibody served as a negative control and subsequent steps were performed in an identical fashion. Cells were washed again and incubated for 30 minutes in 0.8 μg/ml goat, anti-mouse IgG_{2a} conjugated to fluorescein isothiocyanate (FITC) (#M32301, Caltag Laboratories, Burlingame CA) in blocking solution. The washing series was repeated and coverslips were mounted onto microscope slides with 2.5 μl of VectaShield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) to counterstain DNA (H-1200, Vector Laboratories, Burlingame CA). Coverslips were sealed with nail polish and cells were viewed using a Leica DMLB fluorescent microscope. A minimum of 600 cells were evaluated at 400x in random or sequential fields of view. For each field of view, total cell number, total proliferating (PCNA+) cells, total satellite cells (NCAM+), and proliferating satellite cells (NCAM+/PCNA+) were enumerated by two independent evaluators blinded to the identity of the sample. The number of non-myogenic (NCAM-) and proliferating non-myogenic (NCAM-/PCNA+) cells were calculated by difference.

J. Immunostaining for Myogenin

Porcine satellite cells that had been stained for the presence of NCAM (described above) were incubated in undiluted F5D hybridoma supernatant (containing anti-myogenin antibodies) for 1-2 hours (see Table 2.1 for description of antigen). Incubation of 1 μg/ml non-specific mouse IgG in the place of F5D hybridoma supernatant served as a negative control and subsequent steps were performed in an identical fashion. Cells were then incubated with 1:100 goat, anti-mouse IgG conjugated to FITC (F-0257, Sigma Chemical Co., St Louis MO) in blocking solution for 30 minutes. The washing series was repeated and cells were mounted onto microscope slides with 2.5 μl of VectaShield mounting medium containing DAPI counterstain. The slides were sealed with nail polish and viewed using a Leica DMLB fluorescent microscope. A minimum of 700 cells were evaluated using the 400x objective in random or sequential fields of view. For each field of view, total cell number, total satellite cells (NCAM+), and total myogenin positive cells (Myog+) were enumerated by two independent evaluators blinded to the identity of the sample.

K. Statistical Analysis

Data sets were analyzed with Statistical Analysis Software (SAS Institute Inc., Cary NC). Percentage data were converted to a decimal value and transformed as the inverse sin of the square root. A one-way Analysis of

Variance (ANOVA) and Bonferroni multiple comparisons test was used to compare treatment means with a Type I error rate of 5%.

Results

A. Live Weights and Muscle Weights

Live weight increased throughout the experimental period (3.4 kg at 1 week to 89.2 kg at 21 weeks); (Figure 2.1.). *Semitendinosus* (ST) muscle weight increased in proportion to body weight with the most rapid increase in cumulative weight gain occurring between 14 and 21 weeks of age (9.7 g at 1 to 463.7 g at 21 weeks (Figures 2.2.). *Longissimus dorsi* (LD) muscle weight also increased throughout the experimental period (45.0 g at 1 week to 1450.0 g at 21 weeks) with the fastest rate of growth occurring between 7 and 14 weeks of age. At 1 to 7 and 14 to 21 weeks of age comparatively slower rates of growth were observed in the LD (Figure 2.3.).

B. DNA and Protein Contents

Total DNA and protein contents of the ST and LD muscles are presented with the changes in muscle weight in Figures 2.2. and 2.3., respectively. Increases in total DNA and protein of both muscles closely parallel increases in muscle weight. Figure 2.4. shows the calculated number of nuclei accumulated per day for 1 to 7, 7 to 14, and 14 to 21 weeks of age. Myonuclei were assumed to have approximately 7.4 pg of DNA (Doumit et al., 1993) with the majority of DNA present in muscle belonging to myonuclei. Based on Figure 2.4., 18.6%,

36% and 45.3% of myonuclei accumulated within 1 to 7, 7 to 14, and 14 to 21 weeks of age, respectively.

The protein to DNA ratios for the ST and LD are presented in Figure 2.5. From 1 to 7 weeks of age, protein accumulation in the ST and LD occurred proportionately to DNA accretion. However, from 7 to 21 weeks of age, protein accumulated at a greater rate than did DNA in both the ST and LD.

C. Percentage of Satellite Cells (NCAM+ Cells)

Panel B of Figure 2.6. demonstrate representative staining for the presence of NCAM. Closed and open arrows indicate positive and negative staining, respectively. Panel B of Figure 2.9. depicts representative non-specific staining for NCAM. NCAM immunostaining is characterized by intense TRITC staining in a punctate pattern that is confined to the surface of satellite cells. The percentage of NCAM+ cells declined from 57.9% to 33.8% from 1 to 7 weeks of age and remained constant thereafter ($P < 0.05$); (Figure 2.10.). The percentages of satellite cells in coverslips stained for the quantification of differentiating satellite cells (Myogenin+ cells) were similar to the above data (data not shown). Conversely, the percentage of non-myogenic (NCAM- cells) increased from 42.1% in 1-week-old pigs to 66.2% in 7-week-old pigs ($P < 0.05$).

D. Percentage of Proliferating Satellite Cells (NCAM+/PCNA+ Cells)

Panel B of Figure 2.7. depicts representative staining for the presence of PCNA. Closed and open arrows indicate positive and negative staining,

respectively. Panel D of Figure 2.9. demonstrates representative non-specific staining for PCNA. PCNA immunostaining is characterized by intense FITC staining confined to the nuclei of cells. Although there was a decline in the percentage of proliferating satellite cells between 1 and 14 weeks of age ($P < 0.05$), no other differences were detected (Figure 2.11.). A high proportion (79.4%) of satellite cells isolated from the ST muscle of 21-week-old pigs remain in a proliferative (PCNA+) state.

E. Percentage of Proliferating Non-Myogenic Cells (NCAM-/PCNA+ Cells)

The percentage of proliferating non-myogenic cells increased from 38.9% in 1-week-old pigs to 74.1% in 7-week-old pigs ($P < 0.05$); (Figure 2.12.). No differences in the percentage of proliferating non-myogenic cells were detected at 7, 14, or 21 weeks of age.

F. Percentage of Differentiating Satellite Cells

Panel B of Figure 2.8. demonstrates representative immunostaining for myogenin. Closed and open arrows indicate positive and negative staining, respectively. Panel F of Figure 2.9. depicts representative non-specific immunostaining for myogenin. Myogenin staining is characterized by intense FITC staining that is confined to the nuclei of satellite cells. The percentage of differentiating satellite cells were 30.7% in 1-week-old pigs, but decreased to 14.4% in 7-week-old pigs ($P < 0.05$) (Figure 2.13.). The percentage of

differentiating satellite cells continued to decline numerically from 7 to 21 weeks of age.

Discussion

Powell and Aberle (1975) demonstrated that in lines of swine differing in muscularity, increased muscle mass is associated with an increased DNA content. Therefore, it is generally accepted that DNA accretion is essential to muscle hypertrophy and growth. Since those nuclei incorporated into muscle fibers are incapable of DNA synthesis (Stockdale and Holtzer, 1961), DNA accretion must occur by other cellular mechanisms. Satellite cells facilitate DNA accretion through proliferation, followed by differentiation and fusion with existing muscle fibers, thereby increasing the capacity of the muscle fiber to synthesize myofibrillar protein (Moss and Leblond, 1971; Cheek et al., 1970, 1971). The objective of the present study was to characterize the age-related changes in populations of proliferating and differentiating satellite cells in growing pigs from 1 to 21 weeks of age.

It appears that the ST muscle is in a rapid state of growth from 1 to 21 weeks of age and is accommodated by concurrent increases in both total DNA and protein (Figure 2.2). Changes in ST muscle growth closely parallelled changes in total live weight (Figure 2.1.) from 1 to 21 weeks of age, which makes the ST a suitable model in which to study skeletal muscle growth (Dr. Robert Merkel, personal communication). As in the case of the ST muscle, DNA and protein contents of the LD muscle also closely paralleled changes in total muscle

weight, indicating a similar association of muscle growth to DNA and protein accretion (Figure 2.3.). However, the largest gain in LD muscle weight occurred from 7 to 14 weeks of age with relatively slower rates of gain at the early and late phases of growth. The cellular mechanisms contributing to the observed differences in ST and LD muscle growth from 1 to 21 weeks of age are unknown.

Regardless of the observed differences in muscle growth of the ST and LD, both muscles exhibited similar changes in the protein to DNA ratio from 1 to 21 weeks of age (Figure 2.5.). It appeared that from 1 to 7 weeks of age, ST and LD DNA and protein accumulate in a proportional manner. However, at the later stages of growth (7 to 21 weeks) protein accumulated at a greater rate than DNA. This indicates that an individual skeletal muscle DNA unit in these muscles are capable of accommodating a larger protein pool at the later phases of the experimental period.

The proportion of satellite cells as a percentage of the total cell population isolated from the ST muscle declines with age (Figure 2.10.). Other studies for the pig (Campion, 1981), the mouse (Cardasis and Cooper, 1975) and the rat (Allbrook et al., 1971) demonstrated age-related declines in the proportion of nuclei classified as satellite cells. The current study provides the first evidence to indicate that there is an apparent increase in the proportion of non-myogenic (NCAM-) cells to satellite cells (NCAM+) in muscle of growing pigs from 1 to 7 weeks of age.

A number of explanations can account for the observed increase in proportion of non-myogenic cells to satellite cells. A relatively high level of

differentiation at 1 week of age (Figure 2.13.) indicates that a greater proportion of satellite cells are committed to differentiation and may not be extracted from the tissue using the present isolation method. Therefore, as a percentage of total cells isolated from the ST muscle, the proportion of satellite cells would decrease relative to non-myogenic cells. It is possible that changes in the levels of one or more mitogens in muscle may influence the proportion of satellite cells to non-myogenic cells. Vandenburgh et al. (1984) demonstrated that a 16 to 22-hour exposure of embryonic chicken muscle cultures to muscle extracts from 86-day-old mice increased the proportion of fibroblastic cells when compared to extracts from younger (28-day-old) mice. It is interesting to note that the percentage of proliferating non-myogenic cells (NCAM-/PCNA+) also increased at the later time points examined in this study (Figure 2.12.). Mitogens released from crushed extracts of rat muscle have been shown to stimulate the proliferation of satellite cells but not muscle derived fibroblasts (Bischoff, 1986b; Chen et al., 1994). The active mitogen in crushed muscle extract, later determined to be hepatocyte growth factor (HGF) (Tatsumi et al., 1998), has been shown to be developmentally regulated in skeletal muscle (Jennische et al., 1993). It is possible that levels of HGF decrease throughout growth of porcine skeletal muscle or satellite cells become unresponsive to HGF, thereby altering the proportion of myogenic to non-myogenic cells. A number of other growth factors may be capable of influencing the proportions of satellite cells to non-myogenic cells and it is the relative levels of these growth factors in muscle that may govern the *in vivo* proportions of satellite cells to non-myogenic cells.

It is generally accepted that at birth a high proportion of satellite cells exist in a proliferative state and as an animal ages this proportion decreases as satellite cells enter a quiescent state (reviewed by Allen et al., 1979). However, the present study indicates that a relatively modest decline in satellite cell proliferation occurs in the ST muscle of growing pigs (see Figure 2.11.). Furthermore, a high proportion (greater than 75%) of satellite cells remain proliferative at 21 weeks of age. Based on changes in ST muscle weight as well as the rates of DNA and protein accretion, it is apparent that the ST muscle is rapidly growing from 1 to 21 weeks of age (Figures 2.2.) Therefore, a high index of satellite cell proliferation is not unexpected.

A number of studies have reported declines in satellite cell proliferation at earlier ages in the pig (Mulvaney et al., 1988) and during growth in the turkey (Mozdziak et al., 1994). However, these studies report a decline in satellite cell proliferation (utilizing ^{3}H-Thymidine or BrdU to label proliferating cells) as a percentage of total muscle nuclei (which includes both incorporated nuclei and satellite cell nuclei). Since the number of myonuclei per muscle fiber increases throughout postnatal growth (Moss and Leblond, 1971), the percentage of muscle nuclei that are satellite cells would be expected to decrease. Consequently, the DNA labeling index would decline when expressed relative to total muscle nuclei. The findings of the current study are in general agreement with those of Schultz (1996), who reported that 80% of satellite cells from 30-day-old growing rats proliferate with a 32-hour cell cycle as determined by dual-labeling experiments utilizing ^{3}H-Thymidine and BrdU. The remaining 20%

proliferate at a much slower rate, possibly entering a quiescent state between divisions. Although the data of Schultz (1996) do not provide information about changes in satellite cell cycle time at multiple time-points throughout growth, they are in agreement with the high proportion of proliferating satellite cells reported in this study examining rapidly growing pigs.

Proliferating cell nuclear antigen (PCNA) is undetectable in quiescent cells via immunofluorescence when methanol is used as a fixative, and is up-regulated in the G_1 phase of the cell cycle, reaches a peak the S phase then declines during G_2 (Morris and Mathews, 1989; Bravo and Macdonald-Bravo, 1987b). A number of other studies have employed immunostaining against PCNA to quantify the index of proliferation for satellite cells *in vitro* (Johnson and Allen, 1993; Yablonka-Reuveni and Rivera, 1994; Yablonka-Reuveni et al., 1999). Although is it unknown to what extent porcine satellite cells express detectable levels of PCNA throughout the cell cycle, it appears that PCNA expression is correlated to DNA synthesis and is well suited for use as an index of proliferation.

Although not quantified in these studies, some heterogeneous PCNA staining was observed with increasing age. Proliferating cell nuclear antigen staining (PCNA) of 1-week-old pig satellite cells appeared to be fairly homogeneous and intense whereas cells isolated from 21-week-old pigs contained populations of PCNA positive cells with both intense and less intense staining (data not shown). This observation, taken together with data reported by Schultz (1996), indicate that changes may also occur in the proportion of porcine satellite cells that progress through the cell cycle at different rates from 1 to 21

weeks of age. More work needs to be done to determine the nature of this phenomenon and the impact on skeletal muscle growth.

To my knowledge, no information exists on the age-related changes in *in vivo* satellite cell differentiation from birth to maturity in the growing pig. Data reported in this thesis demonstrate that approximately 30% of satellite cells are myogenin positive at 1 week of age and this percentage declines to approximately 8% at 21 weeks of age (Figure 2.13.). It is interesting to note that the majority of DNA accumulates from 7 to 21 weeks of age, a time at which the low levels of satellite cell differentiation exist (Figure 2.4.). It is possible that the high index of proliferation observed within the experimental period serves to increase the absolute number of satellite cells within the muscle. Early studies demonstrated an increase in the absolute number of satellite cells throughout postnatal growth in the *peroneus longus* and *sartorius* muscles of the pig (Campion et al., 1981), the *semimembranosus* muscle of Japanese quail (Campion et al., 1982) and the *soleus* muscle in the rat (Gibson and Schultz, 1983). More recent studies by Yablonka-Reuveni et al. (1999) demonstrate that a larger population of satellite cells (MAPK+ cell) exist in muscle fiber cultures isolated from 10-month-old rats at plating compared to cultures from younger rats. Thus, it appears that a large population of satellite cells may exist in the muscle of older animals. In this case, although the rate of satellite cell differentiation declines within the experimental period, the absolute number of satellite cells that incorporate into the muscle fibers may remain high due to a larger population of satellite cells. In addition, the decrease in the rate of

differentiation may also serve to maintain the population satellite cells capable of proliferation (i.e. those that have not entered the differentiation program and have become post-mitotic) causing a further increase in the absolute numbers of satellite cells. Thus, it is possible to maintain a high level of incorporation of nuclei into a muscle fiber even if a decrease in the rate of satellite cell differentiation is observed.

Myogenin has been shown to be up-regulated in mononucleated myogenic cells relatively early in the differentiation program and remain high until fusion of the cell into a syncytial myotube or muscle fiber (Andres and Walsh, 1996). However, myogenin positive satellite cells retain the capability to synthesize DNA and it is not until later in the differentiation program that these cells become post-mitotic and begin to exhibit the contractile phenotype. At this point it is unclear if satellite cells are capable of undergoing multiple rounds of proliferation after becoming positive for myogenin or if differentiation proceeds immediately after completing the current round of proliferation. However, since myogenin expression indicates a commitment to terminal differentiation, this marker provides a relative indicator of the proportion of cells committed to fusion.

At this time it is impossible to rule out programmed cell death or apoptosis as a possible fate of satellite cells quantified in this study. Apoptosis has been observed in satellite cell lines *in vitro* in response to serum starvation or staurosporine treatment (Mampuru et al., 1996; Maglara et al., 1998; McArdle et al., 1999). However, it is unknown if satellite cells or incorporated myonuclei undergo significant amounts of apoptosis throughout postnatal growth. A

number of recent studies have determined that apoptosis also exists as a possible fate for incorporated myonuclei during development or in response to damage, denervation and disease (Borisov and Carlson, 2000; reviewed by Sandri and Carraro, 1999). Future work in this area will determine the role of programmed cell death or apoptosis in DNA accretion and skeletal muscle growth throughout postnatal growth. Apoptosis may be a mechanism employed to control the size of a satellite cell population containing a high proportion of proliferating and a decreasing proportion of differentiating cells as observed in this study.

It is evident from the above study that satellite cells from the *semitendinosus* muscle of growing pigs retain a high level of proliferation and exhibit an age-related decline in satellite cell differentiation throughout postnatal growth. Therefore, it appears that the differentiation and fusion may be a critical step in regulation of skeletal muscle hypertrophy. Future studies should determine the mechanisms by which satellite cell differentiation is regulated *in vivo* in order to develop efficient growth-promoting agents for the optimization of DNA accretion. Furthermore, the cell cycle time of satellite cells in growing pigs was not established in this study, but may be an additional mechanism by which the activity of satellite cells may be modulated.

Chapter 2

Tables and Figures

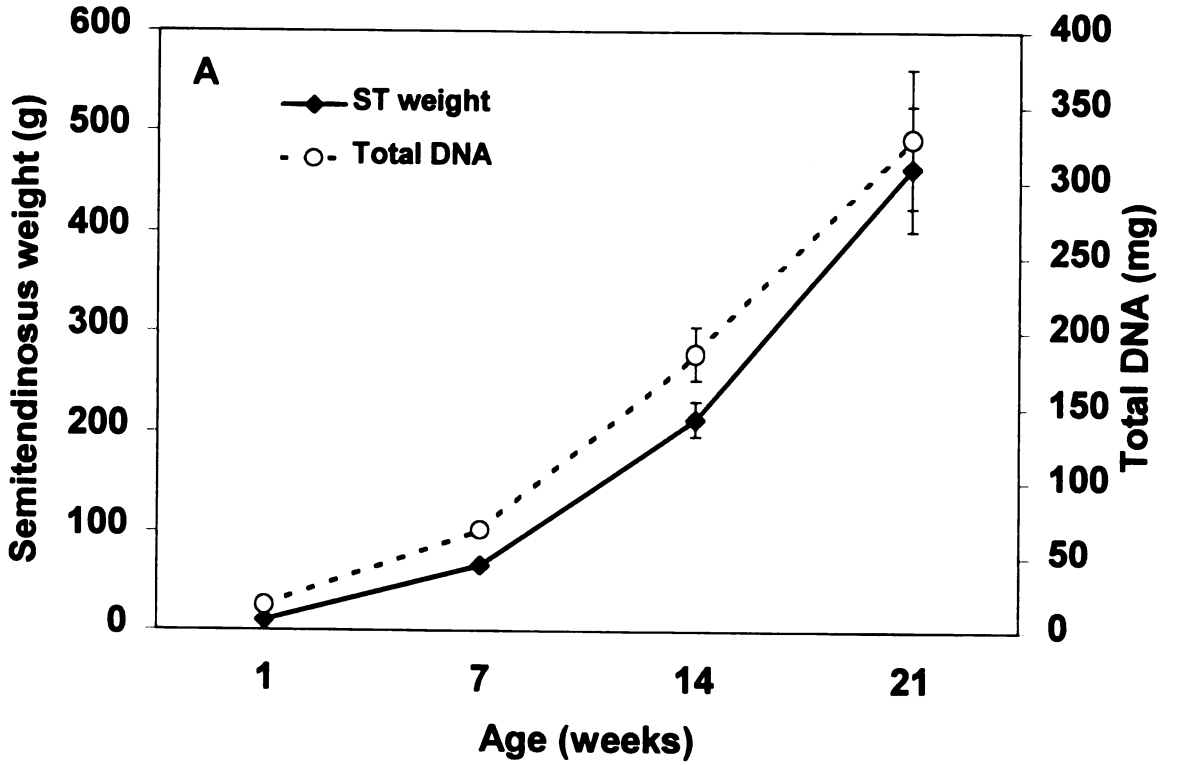
Table 2.1. Antibodies used to quantify porcine satellite cell proliferation and differentiation.

Antibody	Antigen	Antigen Description and Expression	References
5.1H11	Neural cell adhesion molecule (NCAM)	•expressed on cell surface of porcine satellite cells as well as human myoblasts and myotubes. •not expressed on either fetal human skin fibroblasts or muscle fibroblast cells.	Blanton et al., 1999 Baroffio et al., 1993 Walsh et al., 1989 Webster et al., 1988 Walsh and Ritter, 1981
Anti-Proliferating Cell Nuclear Antigen (PCNA), Clone PC10	Cyclin or PCNA	•auxiliary factor to DNA polymerase δ •expression of both mRNA and protein increases during G_1 phase, reaches a maximum in S, then declines during G_2/M phase	Baserga, 1991 Morris and Mathews, 1989 Bravo et al., 1987a Tan et al., 1986
F5D	Myogenin	•member of the muscle-specific basic helix loop helix (bHLH) family of transcription factors •expression occurs early in differentiation program •expression indicates a commitment to differentiation, but can occur prior to exit from cell cycle	Cornelison and Wold, 1997 Andres and Walsh, 1996 Smith et al., 1994

Figure 2.1. Liveweight (kg) of growing pigs at 1, 7, 14, and 21 weeks of age. Error bars represent standard error of the mean (SEM). When not shown error bars are smaller than the marker.


100
90
80
70
60
50
40
30
20
10
0
Live Weight (kg)
0
1
7
14
21
Age (weeks)

Figure 2.2. *Panel A:* left s*emitendinosus* muscle weight (g) and total DNA (mg) of growing pigs at 1, 7, 14, and 21 weeks of age. *Panel B:* left *semitendinosus* muscle weight (g) and total protein (g) of growing pigs at 1, 7, 14 and 21 weeks of age. Error bars represent standard error of the mean (SEM). When not shown error bars are smaller than the marker.


A
ST weight
Total DNA
Semitendinosus weight (g)
Total DNA (mg)
600
500
400
300
200
100
0
400
350
300
250
200
150
100
50
0
1
7
14
21
Age (weeks)

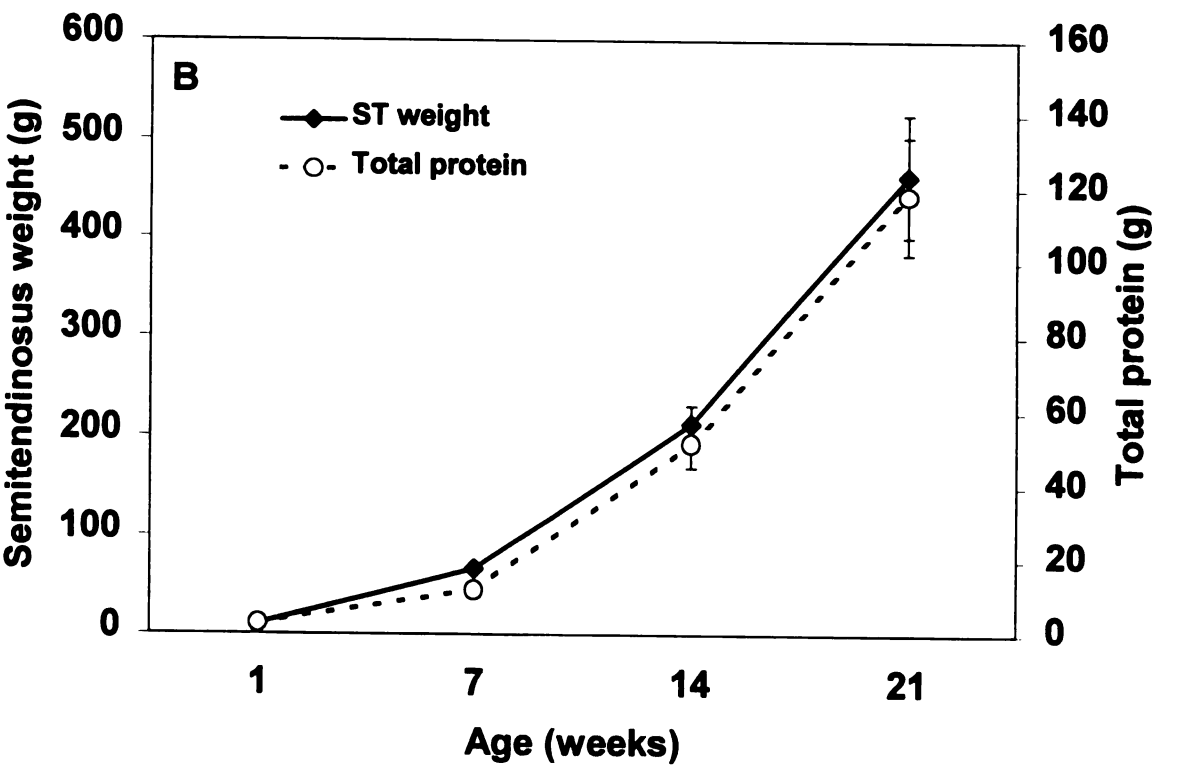
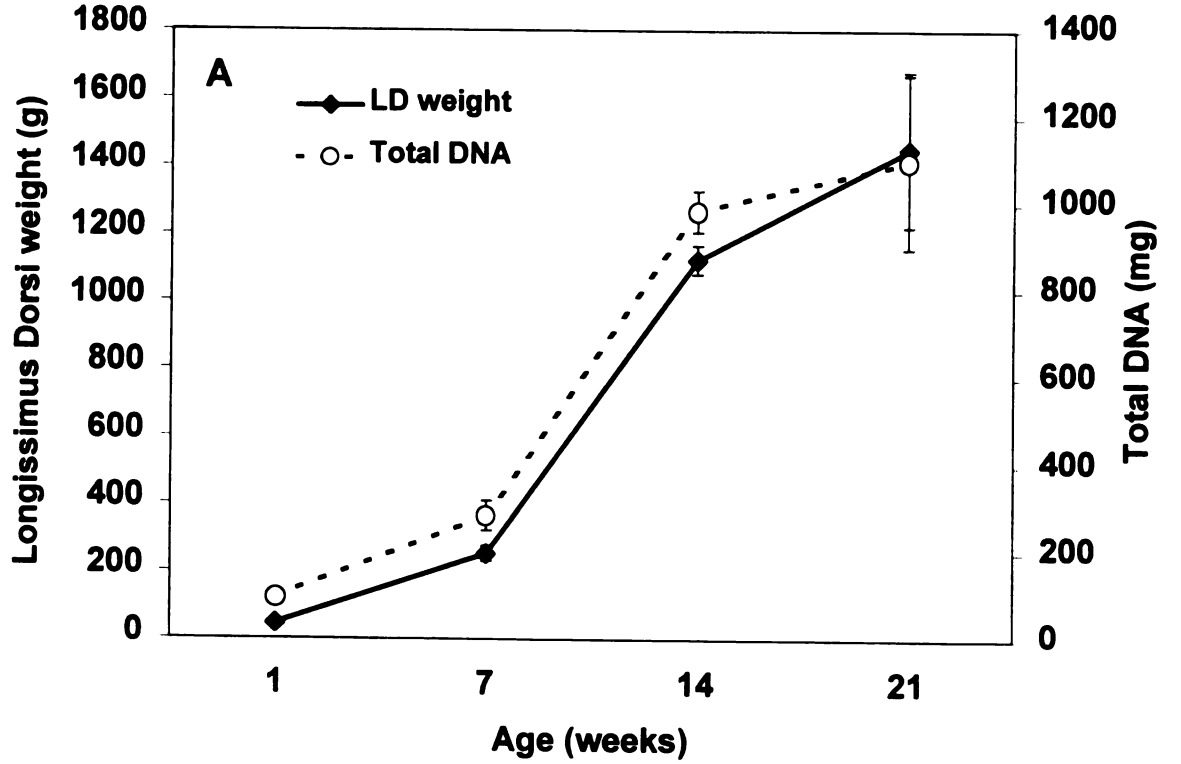

B
ST weight
Total protein
Semitendinosus weight (g)
Total protein (g)
600
500
400
300
200
100
0
160
140
120
100
80
60
40
20
0
1
7
14
21
Age (weeks)

Figure 2.3. *Panel A:* right *longissimus dorsi* muscle weight (g) and total DNA (mg) of growing pigs at 1, 7, 14, and 21 weeks of age. *Panel B:* right *longissimus dorsi* muscle weight (g) and total protein (g) of growing pigs at 1, 7, 14, and 21 weeks of age. Error bars represent standard error of the mean (SEM). When not shown error bars are smaller than the marker.


A
LD weight
Total DNA
Longissimus Dorsi weight (g)
Total DNA (mg)
1800
1600
1400
1200
1000
800
600
400
200
0
1400
1200
1000
800
600
400
200
0
1
7
14
21
Age (weeks)

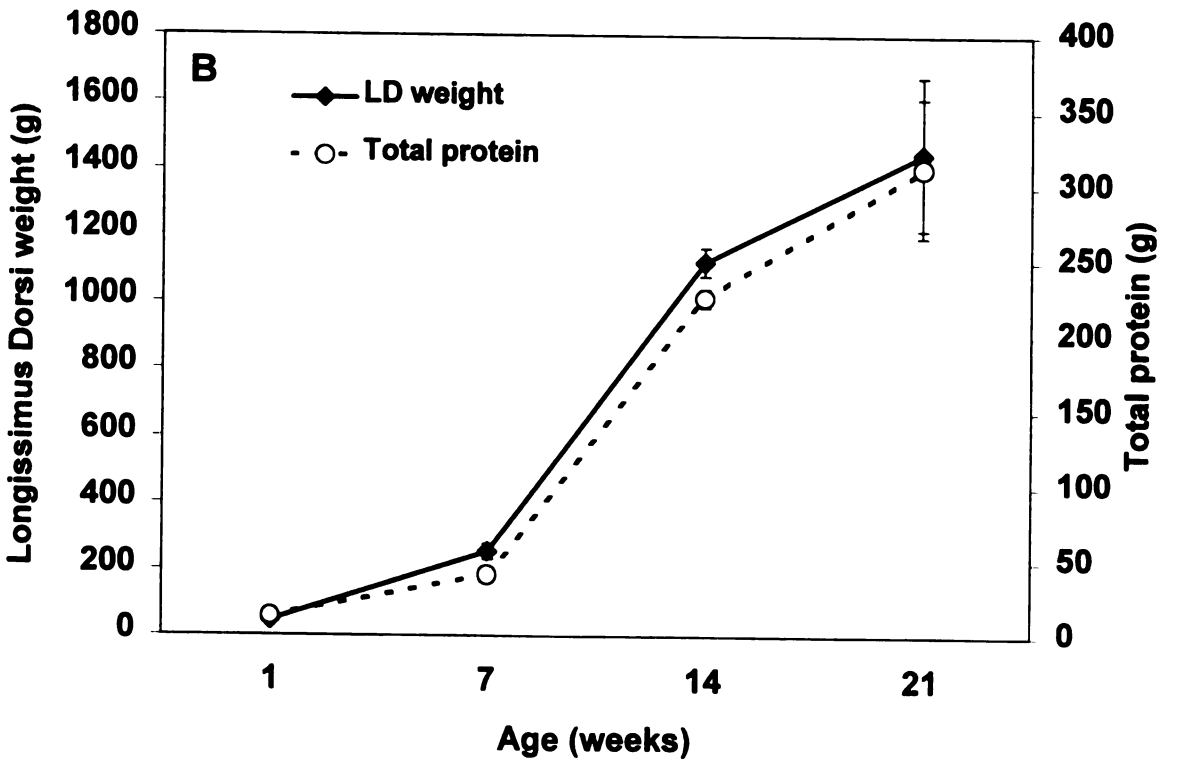

B
LD weight
Total protein
Longissimus Dorsi weight (g)
Total protein (g)
1800
1600
1400
1200
1000
800
600
400
200
0
400
350
300
250
200
150
100
50
0
1
7
14
21
Age (weeks)

Figure 2.4. Daily myonuclear accretion of the left *semitendinosus* muscle of growing pigs from 1 to 7, 7 to 14, and 14 to 21 weeks of age. Number of nuclei accumulated per day calculated based on 7.4 pg DNA/nucleus (Doumit et al., 1993).

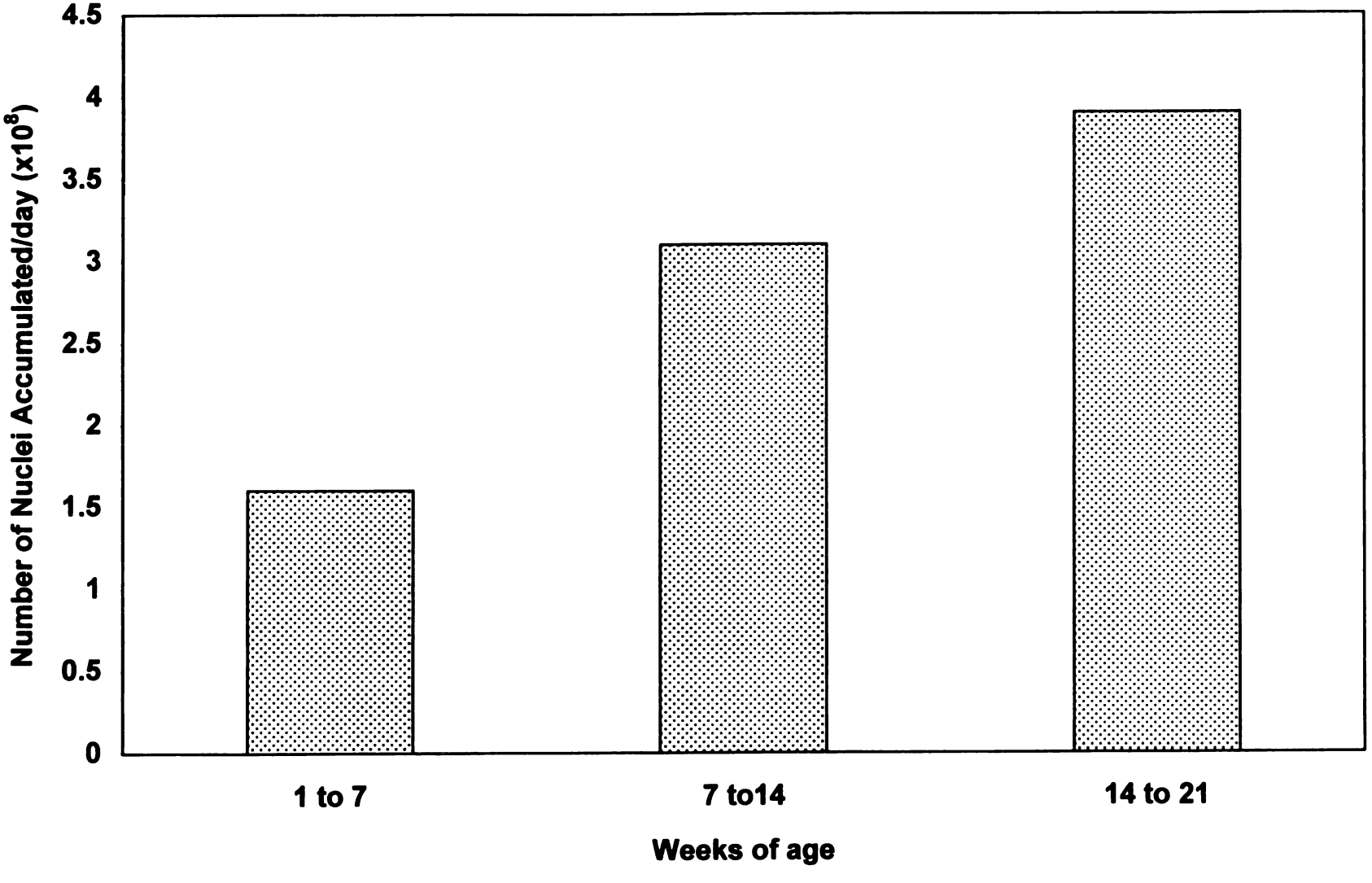

4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Number of Nuclei Accumulated/day (x10^8)
1 to 7
7 to14
14 to 21
Weeks of age

Figure 2.5. Protein to DNA ratio of the left s*emitendinosus* and right *longissimus dorsi* muscles of growing pigs at 1, 7, 14 and 21 weeks of age. Error bars represent standard error of the mean (SEM). When not shown error bars are smaller than the marker.

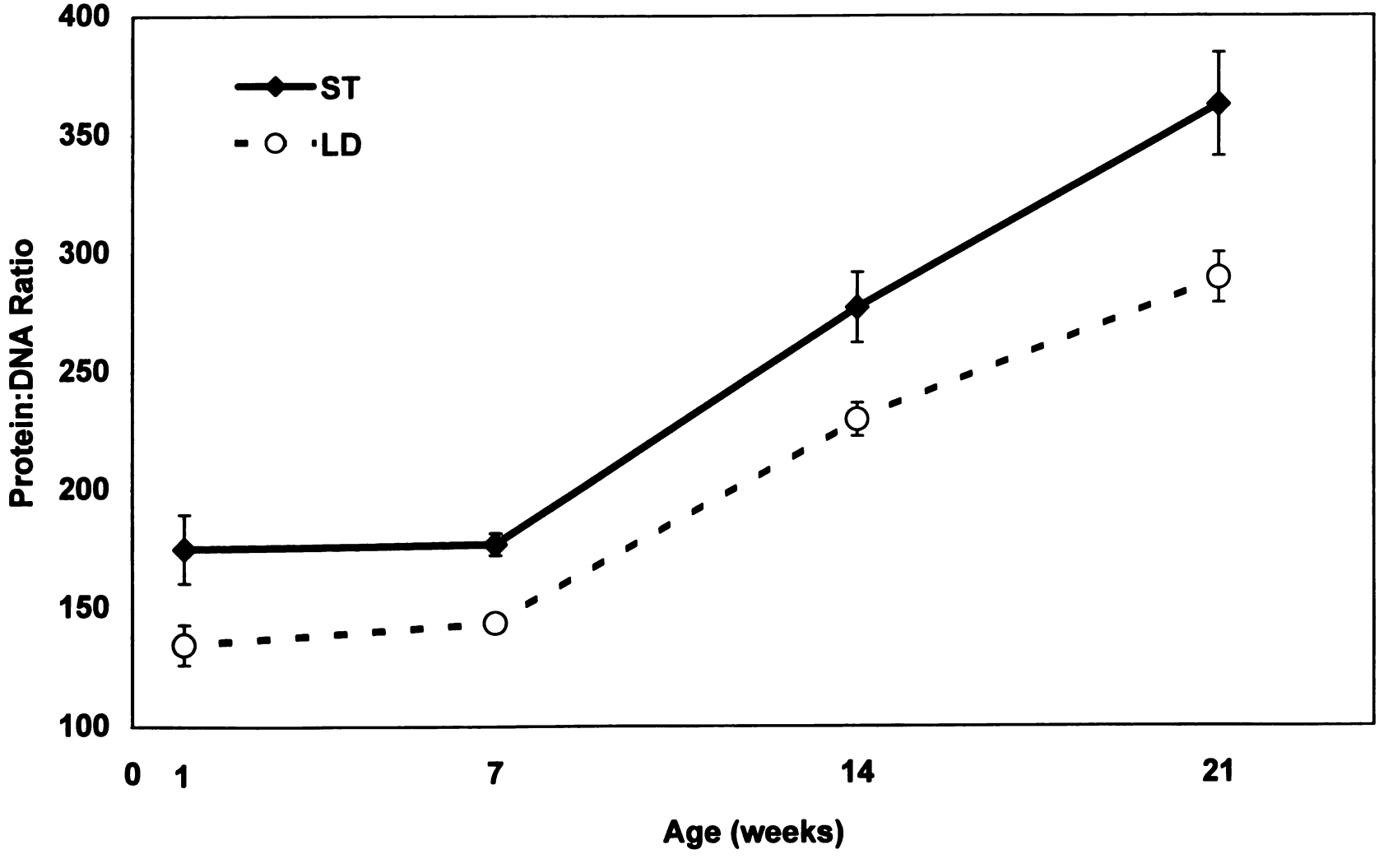

400
350
300
250
200
150
100
Protein:DNA Ratio
ST
LD
0
1
7
14
21
Age (weeks)

Figure 2.6. Fluorescent micrographs of porcine satellite cells stained for the presence of neural cell adhesion molecule (NCAM). Micrographs are presented in color and at 400x magnification. *Panel A:* Detection of total nuclei accomplished by counterstaining cells with 4',6-diamidino-2-phenylindole (DAPI). *Panel B:* Representative immunostaining for NCAM. Closed and open arrows indicate positive and negative staining, respectively.

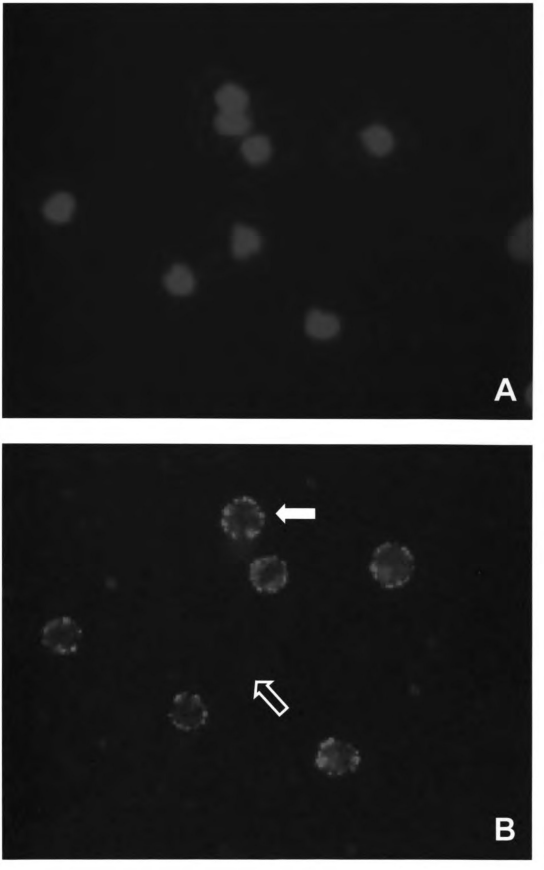

A
B

Figure 2.7. Fluorescent micrograph of porcine satellite cells stained for the presence of proliferating cell nuclear antigen (PCNA). Micrographs are presented in color and at 400x magnification. *Panel A:* Detection of total nuclei accomplished by counterstaining cells with 4',6-diamidino-2-phenylindole (DAPI). *Panel B:* Representative immunostaining for PCNA. Closed and open arrows indicate positive and negative staining, respectively.

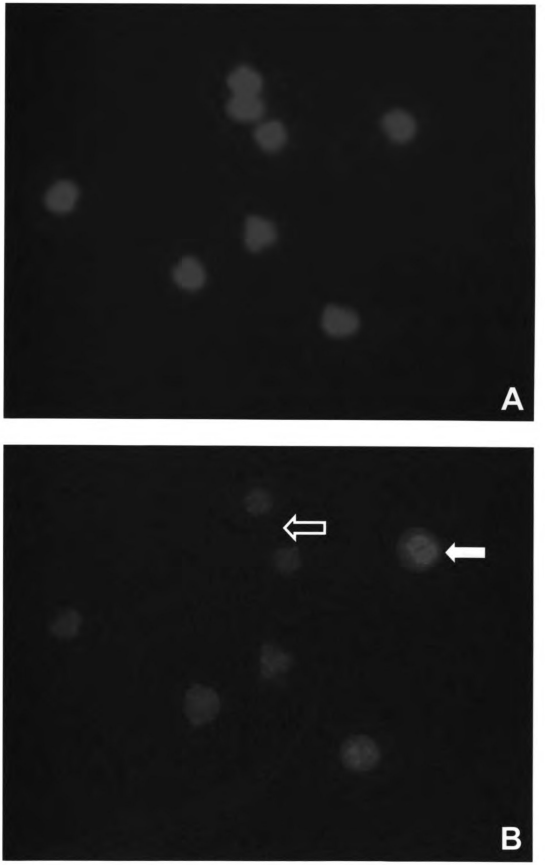

A
B

Figure 2.8. Fluorescent micrographs of porcine satellite cells stained for the presence of myogenin. Micrographs are presented in color and at 400x magnification. *Panel A:* Detection of total nuclei accomplished by counterstaining cells with 4',6-diamidino-2-phenylindole (DAPI). *Panel B:* Representative immunostaining for myogenin. Closed and open arrows indicate positive and negative staining, respectively.

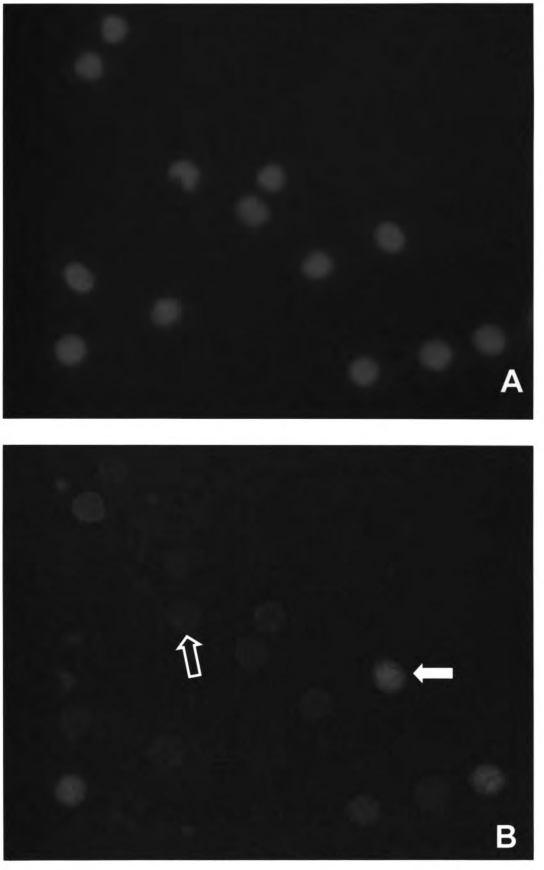

A
B

Figure 2.9. Fluorescent micrographs depicting representative non-specific immunostaining for NCAM, PCNA, and myogenin. Micrographs are presented in color and at 400x magnification. *Panels A, C, and E:* Detection of total nuclei accomplished by counterstaining cells with 4',6-diamidino-2-phenylindole (DAPI). *Panel B:* Representative non-specific immunostaining for NCAM. *Panel D:* Representative non-specific immunostaining for PCNA. *Panel F:* Representative non-specific immunostaining for myogenin.

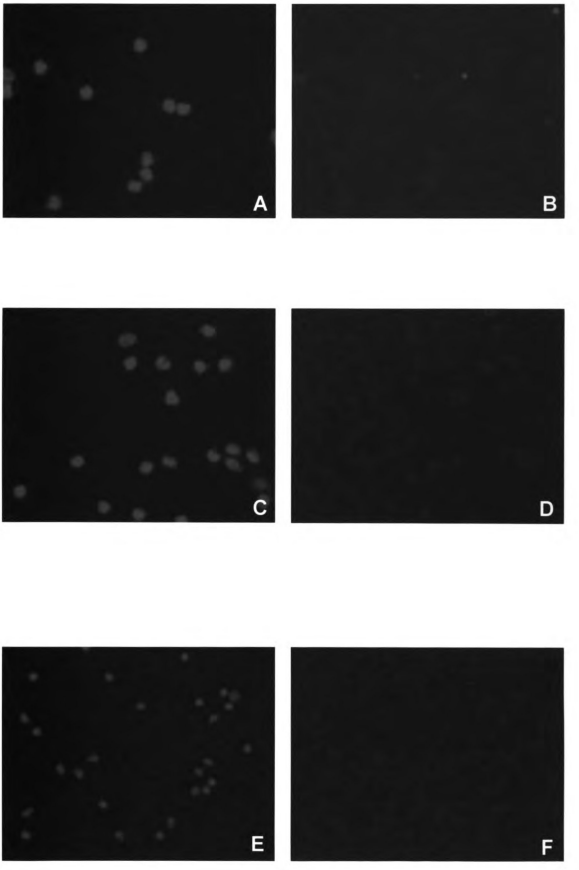

A
B
C
D
E
F

Figure 2.10. Changes in the percentage of satellite cells (cells expressing NCAM) isolated from the right s*emitendinosus* muscle of growing pigs at 1, 7, 14, 21 weeks of age. Error bars represent standard error of the mean (SEM). Different superscripts indicate means that differ *(P <0.05)*.

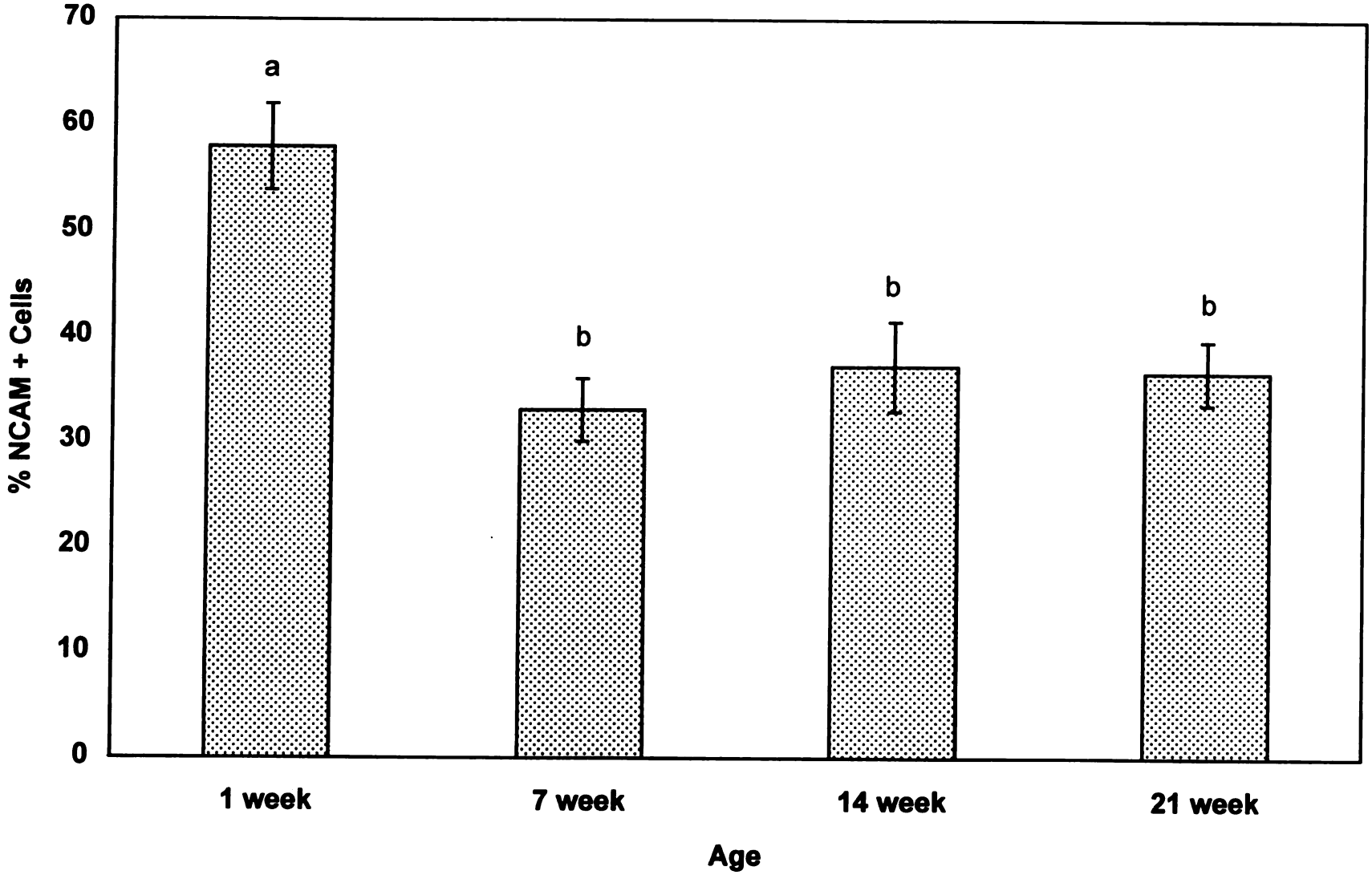

70
60
50
40
30
20
10
0
% NCAM + Cells
a
b
b
b
1 week
7 week
14 week
21 week
Age

Figure 2.11. Changes in the percentage of proliferating satellite cells (cells expressing both PCNA and NCAM) isolated from the right *semitendinosus* muscle of growing pigs at 1, 7, 14, 21 weeks of age. Error bars represent standard error of the mean (SEM). Different superscripts indicate means that differ *(P <0.05)*.

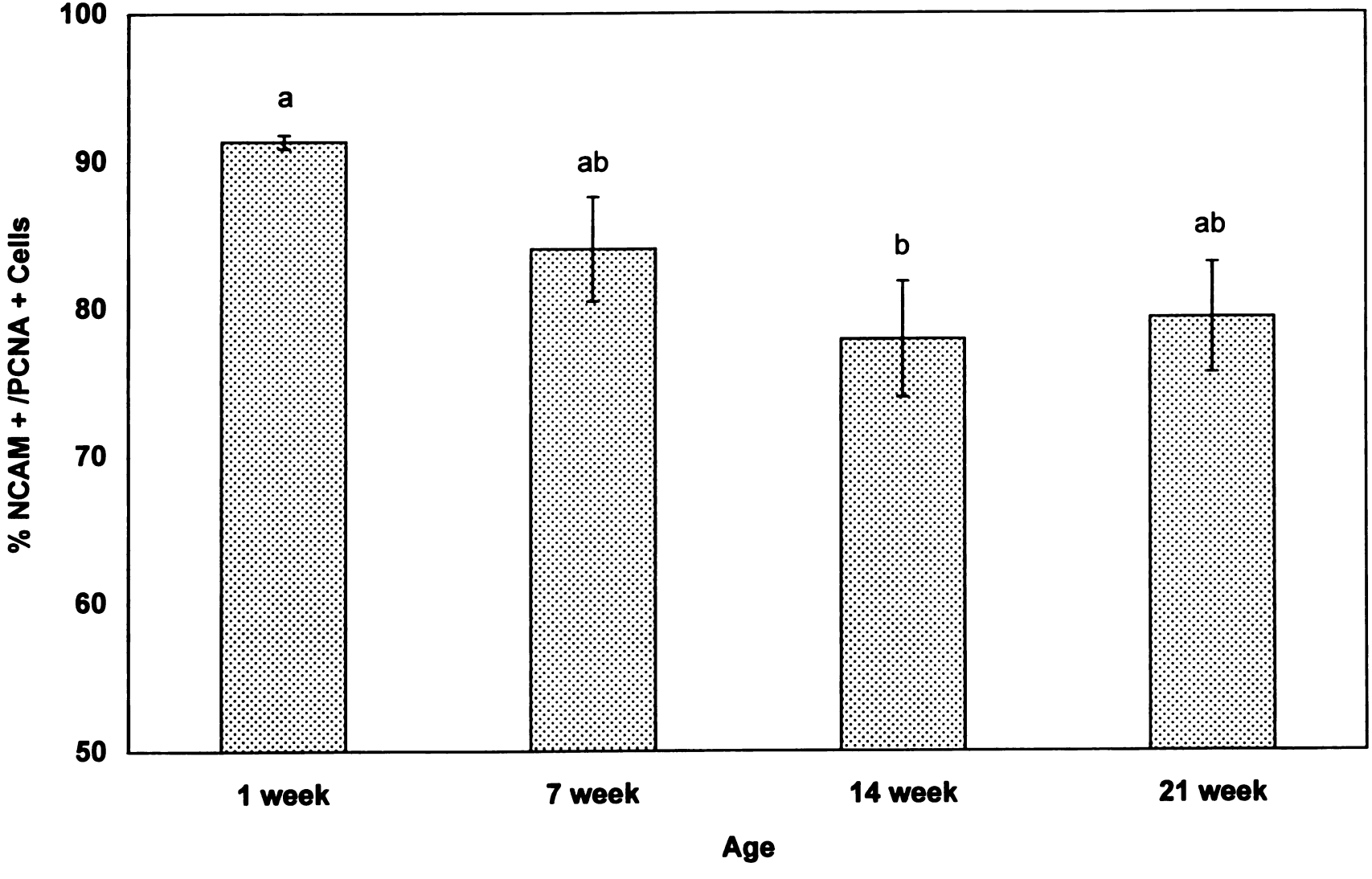

100
90
80
70
60
50
% NCAM + /PCNA + Cells
a
ab
b
ab
1 week
7 week
14 week
21 week
Age

Figure 2.12. Changes in the percentage of non-myogenic proliferating cells (cells expressing PCNA but not NCAM) isolated from the right *semitendinosus* muscle of growing pigs at 1, 7, 14, 21 weeks of age. Error bars represent standard error of the mean (SEM). Different superscripts indicate means that differ *(P <0.05)*.

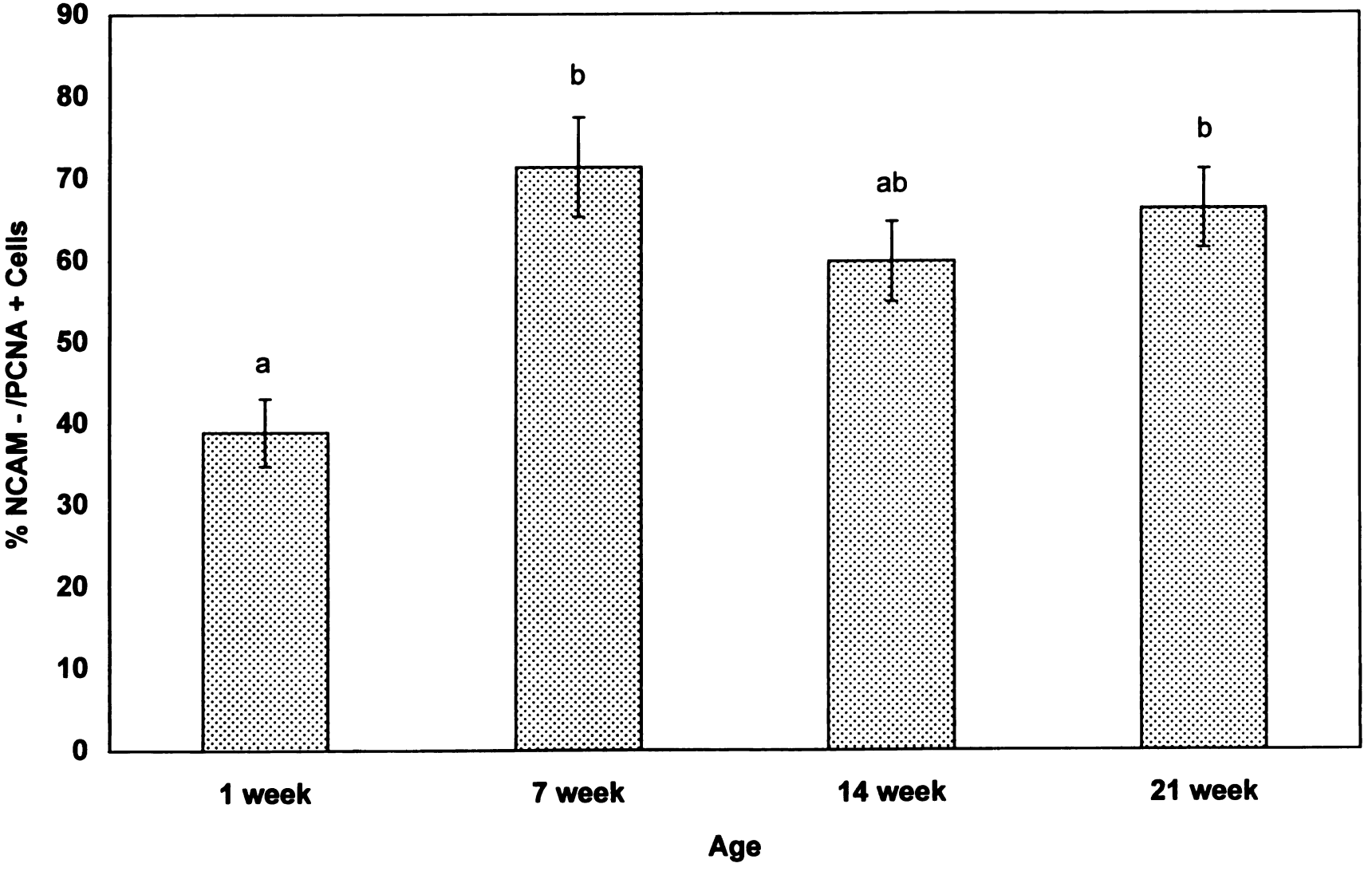
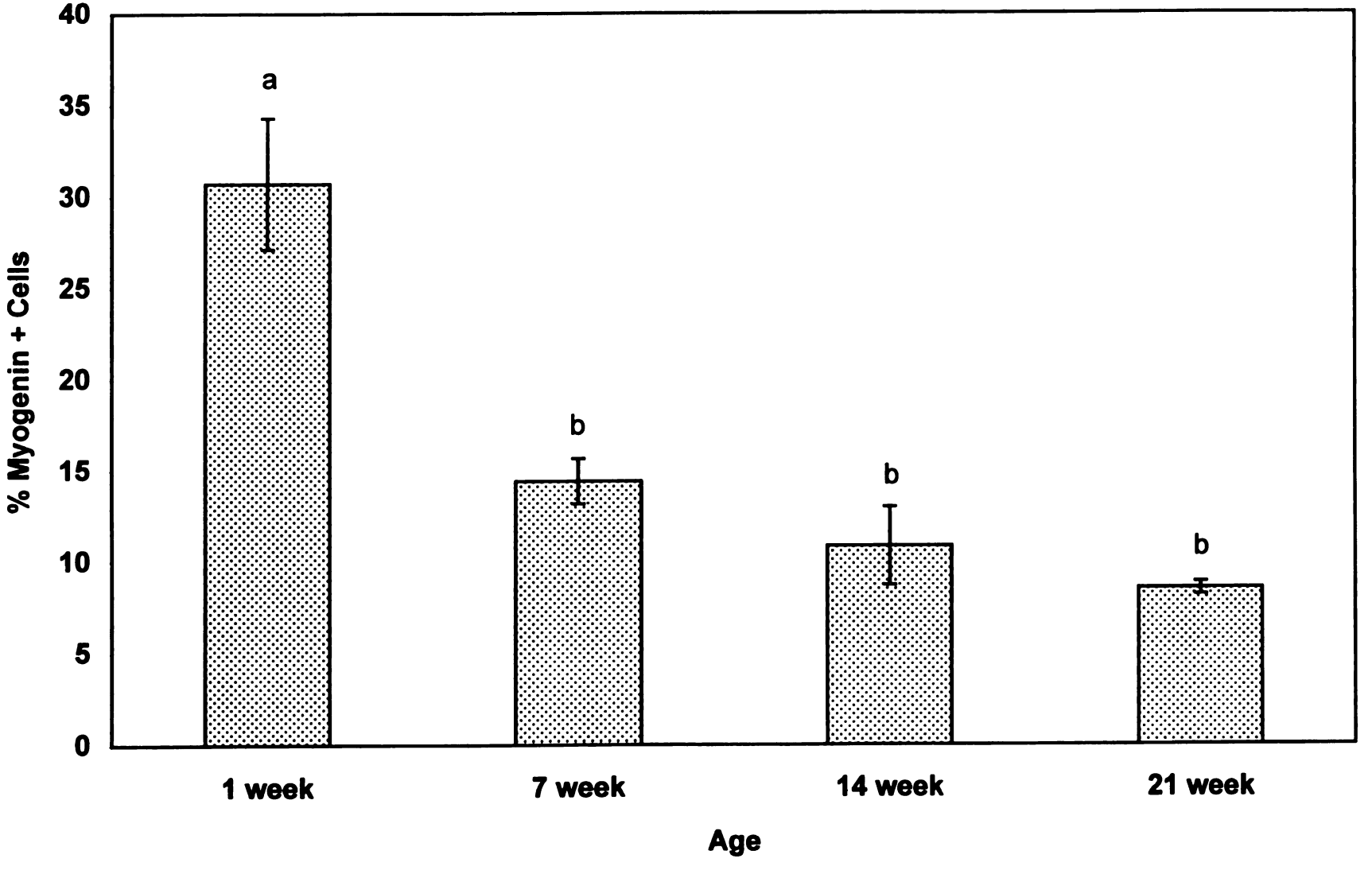

90
80
70
60
50
40
30
20
10
0
% NCAM - /PCNA + Cells
a
b
ab
b
1 week
7 week
14 week
21 week
Age

Figure 2.13. Changes in the percentage of differentiating satellite cells (cells expressing myogenin) isolated from the right *semitendinosus* muscle of growing pigs at 1, 7, 14, 21 weeks of age. Error bars represent standard error of the mean (SEM). Different superscripts indicate means that differ *(P <0.05)*.


40
35
30
25
20
15
10
5
0
% Myogenin + Cells
a
b
b
b
1 week
7 week
14 week
21 week
Age

CHAPTER THREE

RECOMMENDATIONS FOR FUTURE RESEARCH

It appears that in the rapid phases of porcine skeletal muscle growth (1 to 21 weeks of age), satellite cells retain a high level of proliferation (greater than 75% PCNA+ satellite cells) whereas the proportion of satellite cells that are capable of differentiation decline within this period. Furthermore, the percentage of satellite cells to non-myogenic cells isolated from the ST muscle declined from 1 week of age and remained at constant levels thereafter. Thus, the generally accepted theory that satellite cell activity declines as these cells enter a quiescent state does not apply to growth of porcine ST muscle from 1 to 21 weeks of age. Future studies should characterize satellite cell proliferation and differentiation at time-points beyond than 21 weeks of age in order to determine the influence of satellite cell activity at the stages in which ST muscle growth plateaus. Moreover, future studies should examine the changes in satellite cell activity of the LD muscle from 14 to 21 weeks of age. The observed decrease in the rate of growth of the LD at these time-points (Figures 2.3.) would serve as an excellent experimental model to characterize satellite cell activity at a point in time where a relatively slower rate of muscle growth is observed.

Since PCNA is expressed in multiple stages of the cell cycle (Morris and Mathews, 1989), it is important to note that the current immunostaining system used to quantify proliferating satellite cells does not provide information about cell cycle time of PCNA positive nuclei. The possibility arises that changes in

proliferation due to age may occur from a lengthening cell cycle and not from changes in the number of cells progressing through the cell cycle at any time. Bischoff (1986b) observed that satellite cells have a cell cycle time of approximately 12 hours when exposed to mitogens released from an extract of crushed muscle *in vitro*. On the other hand, Schultz (1996) demonstrated that 80% of satellite cells from growing rat muscle proliferate with an estimated 32-cell cycle time with the remaining 20% proliferating more slowly. Taken together, these data indicate that cell cycle time may be another regulatory checkpoint for satellite cell activity and that populations of satellite cells with different proliferative potentials may exist in growing muscle. Future studies should examine if changes in satellite cell cycle time occur from 1 to 21 weeks of age as well as the effect of these changes on skeletal muscle hypertrophy.

The mechanisms responsible for the changes in satellite cell activity observed in the preceding chapter are unclear. The IGF-I system may play a major role in the regulation of satellite cell activity. Insulin-like growth factor-I is unique because it is able to stimulate both proliferation and differentiation of myoblasts (reviewed by Florini et al., 1996). Insulin-like growth factor-I is also demonstrated to act synergistically with FGF-2 to stimulate both proliferation of porcine satellite cell clones (Doumit et al., 1993) and differentiation of rat satellite cells (Allen and Boxhorn, 1989). Possible mechanisms by which IGF-I may elicit a decline in differentiation are 1) levels of IGF-I protein in skeletal muscle decline with age, 2) levels of circulating or locally produced IGFBPs increase with age, 3) levels of IGFR1 on the surface of satellite cells decline with age, 4) the affinity of

IGFR1 for its ligands decline with age, and/or 5) other growth factors that inhibit the myogenic actions of IGF-I increase with age.

Mitogenic growth factors, such as FGF and HGF, may also be responsible for the high degree of proliferation observed in this study. It is unknown if levels of these other growth factors decrease with age in porcine muscle and if the bioactivity of such peptides changes with development. Furthermore, it is unknown if these other growth factors interact with IGF-I to elicit the observed *in vivo* levels of proliferation.

In order to elucidate the mechanisms governing skeletal muscle hypertrophy, it will be necessary to further characterize the proliferating and differentiating populations of satellite cells. Furthermore, it will be important to gain insight into the hormonal control of satellite cell activity, especially control by locally produced growth factors. Such information will be useful in determining the role of satellite cell proliferation and differentiation in DNA accretion of skeletal muscle, ultimately leading to the development of strategies to optimize accretion of lean tissue in meat animal species.

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