To my future patients
ACKNOWLEDGMENTS

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My mother Mary, sister Megan, and loving boyfriend John Michael offered me endless support outside of the lab, which made my attainment of this degree possible. Each of these individuals believed in my dream of becoming a veterinarian and I hope that I can make them proud.
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Satellite cells or muscle stem cells are responsible for postnatal muscle growth and repair.

Satellite cells in mature muscle reside in a quiescent state outside of the cell cycle. Upon proper stimulus, satellite cells become activated, proliferate, and fuse into muscle. Myoblast cell lines are often utilized to simulate satellite cell biology. The 23A2RafERDD2 embryonic mouse myoblasts enter an induced quiescent state upon high levels of Raf stimulation. Approximately 30% of 23A2RafERDD2 myoblasts express Pax7, a satellite cell unique transcription factor. This cell line heterogeneously exhibits varying myogenic regulatory factor combinations, representing all stages in the development of a myoblast. The 23A2RafERDD2 myoblasts, like satellite cells, possess a potential for adoption of divergent fates through asymmetric cell division. The 23A2RafERDD2 myoblasts are an acceptable model for some aspects of satellite cell biology.

Previous work identified two proteins that display a nuclear translocation upon high levels of Raf induction, E2F5 and LEK1. These proteins also were present in the nuclei of quiescent satellite cells in vitro. In vivo work identified these proteins in satellite cells and myonuclei from murine and bovine tissue samples. Differential protein expression patterns were also demonstrated with age. E2F5 and LEK1 expression levels fluctuate from low in a young animal to higher in an adult animal once muscle growth ceases. Aged muscle displayed the lowest levels of expression.
E2F5 and LEK1 play unique roles in entrance and maintenance of states of quiescence and cell cycle exit. The two proteins also exhibit different localizations upon the assignment of divergent fates within satellite cells.
Satellite cells, or muscle stem cells, reside in adult muscle as a small percentage of the nuclear population. Due to unique regenerative properties, satellite cells are able to self-renew and contribute to muscle growth and repair. Upon mitogenic signals, satellite cells activate, proliferate, and fuse into muscle fibers, resulting in hypertrophy. Such hypertrophy promises great advantages to producers. Satellite cells can be identified by a unique protein marker, Pax7.

Embryonic myoblasts serve as a potential model system for satellite cell biology. Previous work from this lab developed a particular murine embryonic cell line, 23A2RafER\textsuperscript{DD2} myoblasts. These myoblasts contain a modified estrogen receptor fused to the Raf molecule. Dose-dependent treatment with 4-hydroxytamoxifen (4HT), an estrogen analog, affects proliferation in the myoblasts. High levels of induced Raf send these 23A2RafER\textsuperscript{DD2} myoblasts into a reversible quiescent state, similar to the quiescent state in which non-active satellite cells reside (Reed et al., 2007). Approximately 30% of the 23A2RafER\textsuperscript{DD2} population expresses Pax7, lending credibility for use of this cell line as a model for satellite cell activity. Reed et al. (2007) identified two proteins, E2F5 and LEK1, which exhibit nuclear translocation upon high levels of induced Raf in 23A2RafER\textsuperscript{DD2} myoblasts, possibly playing roles in induction of this quiescent state.

- **Objective 1:** Characterize the 23A2RafER\textsuperscript{DD2} myoblast population as a model for satellite cell biology.

- **Objective 2:** Establish the relationship between the expression of E2F5 and LEK1 proteins and cell cycle exit \textit{in vivo} via examination of different species and age groups.
CHAPTER 2
MATERIALS AND METHODS

Cell Culture

Stock cultures of 23A2RafER^{DD2} myoblasts, C2C12 myoblasts, and C3H10T1/2 fibroblasts were passaged at about 70% confluency on 10cm plastic plates. 23A2RafER^{DD2} myoblasts required basal medium eagle (BME) media supplemented with 15% v/v fetal bovine serum, 1% v/v penicillin/ streptomycin, 1% v/v L-glutamine, 0.1% gentamycin, and 10nM puromycin. C2C12 myoblasts were cultured in high glucose Dulbecco’s Modified Eagle Medium (D-MEM) with L-glutamine and supplemented with 10% v/v fetal bovine serum, 1% v/v penicillin/ streptomycin, and 0.1% gentamycin. 23A2RafER^{DD2} and C2C12 cells were cultivated on plates coated with 0.1% w/v gelatin. C3H10T1/2 cells utilized uncoated plates and basal medium eagle (BME) media supplemented with 10% v/v fetal bovine serum, 1% v/v penicillin/ streptomycin, 1% v/v L-glutamine, and 0.1% gentamycin reagent solution. All cells were cultured at 37°C in 5% CO₂. Culture media and reagents were purchased from Invitrogen (Carlsbad, CA). Immunofluorescence utilized cells plated at a density of 3.5 x 10^4 on 35mm glass-bottomed plates (World Precision Instruments, Sarasota, FL) coated with 10% v/v BD Matrigel Matrix HC (BD Biosciences, San Jose, CA) in BME.

Western Blots

For the isolation of total cellular proteins, cells were washed with phosphate buffered saline (PBS) and lysed in 4X SDS PAGE sample buffer. Lysates were briefly sonicated, heated at 95°C for five minutes, and stored at -20°C. Equal amounts of protein were loaded into lanes and separated through 10% polyacrylamide gels under denaturing conditions. Proteins were transferred to NitroBind nitrocellulose (GE Water & Process Technologies, Minnetonka, MN) by semi-dry electrotransfer (large semiphor transphor unit, Amersham Pharmacia Biotech AB, San
Francisco, CA). Non-specific antigen binding was blocked by incubation for a minimum of 30 minutes at room temperature in a solution of 5% w/v non-fat dry milk (NFDM) in Tris buffered saline with Tween 20 (TBS-T; 10mM Tris, pH 8.0, 150 mM NaCl, 0.1% v/v Tween 20). Antibodies were diluted in this blocking solution. Blots were next incubated in 1:5 cultured Pax7 supernatant (Developmental Hybridoma Bank, Iowa City, IA) for one hour, followed by three five-minute washes in TBS-T. Peroxidase-labeled goat anti-mouse IgG (H+L) (Vector Laboratories, Burlingame, CA) was used at a 1:2000 concentration as the secondary antibody for 45 minutes, followed by three five-minute washes in TBS-T. Tags were detected via enhanced chemiluminescence (Amersham Biosciences, Picataway, NJ) and developed onto X-ray film.

**Immunocytochemistry**

Fresh, proliferating cells were rinsed free of media with PBS and fixed in 4% v/v paraformaldehyde in PBS for 20 minutes at room temperature and subsequently washed with PBS. Cell membranes were permeabilized for 20 minutes by incubation with 0.1% v/v TritonX-100 in PBS. Non-specific antigens binding sites were blocked with a 60 minute incubation in 5% v/v horse serum in PBS. Individual primary antibodies, or a combination of two primary antibodies derived from different species, were diluted in the 5% horse serum solution as described in Table 2-1. Cells were incubated in the primary antibody solution for 60 minutes at room temperature, followed by three five-minute washes. Goat anti-mouse or anti-rabbit 488/568 Alexafluor antibodies (Molecular Probes, Carlsbad, CA) were diluted 1:250 in blocking solution along with Hoechst dye (1µg/mL final concentration) and added to plates for 45 minutes. Washes with PBS prepared the cells for viewing. Glass bottomed plates allowed for magnification up to 630x with an Eclipse TE 2000-U stage microscope (Nikon, Lewisville, TX) equipped with an X-Cite 120 epifluorescence illumination system (EXFO, Mississauga, Ontario,
Images were captured using a DXM 1200F digital camera and analyzed through the use of the NIS-Elements software (Nikon, Lewisville, TX).

**Immunohistochemistry**

The *tibialis anterior* (TA) muscle of three young (3-4 week old) and three adult (9 months of age) mice were removed, placed in OCT freezing medium (Fischer Scientific, Hampton, NH), frozen by exposure to isopentane cooled by dry ice, and stored at -80°C. Bovine samples consisted of isolated *infraspinatus* (INF) muscle from four young (less than three months of age) animals and four old (11-12 years old) animals. These 1cm x 1cm x 1cm portions were frozen in an identical manner to the murine samples. Cryosections were cut at 8µm, with the exception of aged bovine samples, which were cut at 12µm and affixed to frost resistant slides (Fischer Scientific, Hampton, NH). For each animal and protein combination analyzed, two separate observations were made.

As with immunocytochemistry, cryosections were incubated in 5% horse serum in PBS for 20 minutes at room temperature to block non-specific antigen binding sites. Samples were then incubated with solitary or paired primary antibodies used at dilutions recommended in Table 2-1 for 60 minutes. Combinations included Pax7/E2F5, Pax7/LEK1, dystrophin/E2F5, and dystrophin/LEK1. Three five-minute washes in PBS were followed by the addition of the appropriate secondary antibody. Pax7 immunostaining also was attempted using a M.O.M. (mouse on mouse) immunodetection kit (Vector Laboratories, Burlingame, CA).

Fiber typing was achieved through a 20 minute block followed by a 60 minute incubation in either type I or type IIa myosin heavy chain antibody. Goat biotinylated anti-rat IgG (H+L) (Vector Laboratories, Burlingame, CA), was applied at a 1:100 dilution for 20 minutes. Final detection was possible with the addition of Alexa Fluor-568 streptavidin (Molecular Probes, Carlsbad, CA) at a 1:500 dilution for 45 minutes. Slides were washed, coverslipped, and viewed.
Images were captured and analyzed with the equipment and software mentioned for immunocytochemistry.

**E2F5 Preabsorption**

The column was composed of approximately 50mg of glutathione 4B sepharose beads (Amersham Biosciences, Picataway, NJ) that were hydrated with 250μl TBS (pH 8.0) then collected by centigugation (1000xg for less than 20 seconds). Beads were washed three times with 250μl TBS. To renature the E2F5-GST (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), 25μl of this sample was added to 1mL of TBS, vortexed, and allowed to set on ice for 30 minutes. The renatured GST-E2F5 was added to the glutathione sepharose beads and rotated at 4°C for 30 minutes. Beads were sedimented and supernatant was removed. Beads were washed as above, three times with TBS. Anti-E2F5 antibody diluted at a working concentration of 1:100 (250μl) was incubated with the E2F5-conjugated glutathione sepharose beads on ice for 60 minutes, with mixing every 10 minutes. Supernatant was collected and substituted for primary antibody.

**Statistical Analysis**

The species and age-wise comparison portion of this study was performed in a 2 x 2 factorial design. The experimental units (n) included three murine samples of identical genetic background and four bovine samples from similar genetic background for each age group. Means of myonuclei expressing E2F5 and LEK1 were tabulated and compared using the PROC GLM statement in SAS (SAS Institute, Inc., Cary, NC, 2002). Data for Pax7 positive cells was sorted and analyzed by species. Age and species were the fixed effects. Individual animals were included in the random statement. Pair-wise comparisons between the least square means of the factor levels were assessed through using the LSMEANS statement with the PDIFF option.
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CHAPTER 3
LITERATURE REVIEW

Satellite Cells: Muscle Stem Cells

Muscles produce force and movement in the body. Grouped into units called fasicles by the basal lamina, skeletal muscle fibers span the length of muscle as long, narrow, multinucleated cells. The plasma membrane, or sarcolemma encases these fibers into groups of myofibrils. Myofibrils are composed of bundles of contractile proteins, organized into the sarcomere, the smallest unit to produce force and movement. Repeating arrangements of thick and thin filaments are present in a cross striation pattern. This arrangement allows for dynamic conversion of chemical to mechanical energy by utilizing a cross bridge motion. These units compose a differentiated, highly specialized muscle tissue (Lieber, 1992).

Myogenesis defines the process in which muscle develops, either from an embryo, or during muscle growth and regeneration. Myoblasts precede muscle fibers, or myofibers, in the developmental pathway. These mononucleated muscle precursor cells fuse to form multinucleated myotubes. Maturation of myotubes ultimately leads to the development of muscle fibers and muscle tissue. Adult muscle withstands constant abuses yet regenerates efficiently due to satellite cells (Lieber, 1992).

Alexander Mauro discovered and defined satellite cells from electron micrograph studies of frog skeletal muscle in 1961. Satellite cells, or muscle progenitor cells, lie beneath the basal lamina but above the sarcolemma of muscle fibers. The term satellite cell indicates cell position, satellite and separate from the myofiber itself. Satellite cells compose 2-7% of fiber-associated nuclei, varying by age and muscle group. The small percentage of these cells that exist within muscle, presents difficulties for in vivo studies. Gibson and Schultz (1982) found a higher
percentage of satellite cells in slow soleus muscle than in fast tibialis anterior or extensor digitorum longus muscles.

Resting satellite cells exist in a quiescent state, described by cells that are neither dividing nor terminally differentiated. The single nuclei of these cells contain a high ratio of heterochromatin to euchromatin. Quiescent satellite cells appear reduced in volume and contain few organelles (Schultz, 1976). Chromosomes present in a highly condensed state and the lack of organelles necessary for normal function establishes the inactive state of these cells. Exit and re-entry into this quiescent state without differentiation, along with a certain plasticity define satellite cells as muscle stem cells.

Satellite cells appear after primary muscles have formed, about 17.5 days after birth in mice. Most of these muscle stem cells originate from Pax3/ Pax7 positive fetal muscle progenitor cells (Relaix et al., 2005). Kassar-Duchossoy et al. (2005) demonstrate a somitic origin for satellite cells. However, Seale and Rudnicki (2000) suggest that they are derived from the dorsal aorta. De Angelis et al. (1999) proved that satellite cells express some endothelial makers and could originate from an endothelial source such as the dorsal aorta.

**Characterizing Satellite Cells**

Protein markers can be used to characterize satellite cells. Examples include M-cadherin, a calcium dependent adhesion molecule, desmin, a structural membrane protein, and c-met, the receptor for hepatocyte growth factor (Hawke and Gary, 2001). Myocyte nuclear factor (Hawke and Gary, 2001), VCAM1 (Jesse et al., 1998), Syndecan 3/4 (Cornelison et al., 2001), stem cell antigen-1 (Scimè and Rudnicki, 2006), and CD34 (Beau champ et al., 2000) also mark satellite cells. Through testing for combinations of these proteins, satellite cells can be elucidated. Pax7 functions well as a marker for satellite cells, found in quiescent and activated nuclei (Seale et al., 2000). Immunocytochemistry provides a clear representation of many of these protein markers.
Differences in protein markers suggest heterogeneity in the satellite cell population. Bimodal activity of satellite cell studies in vivo also contribute to this theory. A stretching model to examine activation revealed distinct characteristics. Satellite cells showed differential thresholds by entering the cell cycle at two distinct times. The literature insinuates different subsets of satellite cells, with plasticity ranging from naïve to more committed (Wozniak et al., 2005). Stem cells other than satellite cells contribute to muscle repair at a lesser degree. For example, bone marrow-derived stem cells can participate in muscle regeneration and also contribute to the satellite cell population (LaBarge and Blau, 2002). This finding places satellite cells as intermediates in a stem cell hierarchy.

**Myogenic Regulatory Factors**

The Myogenic Regulatory Factor (MRF) family contains four members: Myf5, MyoD, myogenin, and Mrf4. These basic helix-loop-helix (bHLH) transcription factors control the development of the muscle lineage. The conserved basic DNA binding domain allows for sequence specific binding. Heterodimerization requires the helix-loop-helix region (as reviewed by Sabourin and Rudnicki, 2000).

In the 1987, Davis et al. successfully isolated the MyoD gene from subtractive hybridization procedures using myoblast specific cDNA libraries. This protein proved the ability to convert fibroblasts to cells of a myogenic lineage. Similar methods identified the other three MRFs, all capable of converting other lineages to a myogenic one through over-expression (Sabourin and Rudnicki, 2000).

Satellite cells in the quiescent state do not express MRFs, which suggests a stem cell lineage distinct from myoblasts (Cornelison and Wold, 1997). Upon activation in vitro, an up-regulation of MyoD, a myogenic regulatory factor, is evident within 12 hours (Yablonka-
Reuveni and Rivera, 1994). Activated satellite cells have been shown to express Myf5 (Cornelison and Wold, 1997).

Myf5 and MyoD are considered primary MRFs and contribute to cell commitment and myoblast formation. During myogenesis, expression of MyoD depends on Myf5 or Pax3, a paired box transcription factor (Tajbakhsh et al., 1997). MyoD-null mice exhibit a normal muscle phenotype and a four-fold compensation of Myf5 (Rudnicki et al., 1992). Deletion of this gene also severely impairs muscle regeneration (Megeney et al., 1996).

Myf5-null mice demonstrate a similar phenotype and die perinatally due to rib defects. Myogenin is able to rescue rib formation at this locus (Braun et al., 1992) but cannot completely compensate for its absence in determination (Wang and Jaenisch, 1997). Cells devoid of these two genes die at birth with an absence of skeletal myoblasts and muscle (Rudnicki et al., 1993). Unique functions demonstrate differences among the MRFs. Rudnicki et al. (1993) have shown that MyoD/Myf5 double knockout mice exhibit a complete loss of skeletal muscle and that progenitor cells remain in a multipotent state, contributing to other lineages in the limbs and trunk.

Charge and Rudnicki (2004) suggest that Myf5 plays a role in the self-renewal of satellite cells. They hypothesize that the MyoD-/Myf5+ state of satellite cells provides the model for this theory. However, the return to a quiescent state may occur with a down-regulation of Myf5, due to findings by Cornelison and Wold (1997).

Differentiation and formation of a differentiated myocyte result from the actions of the secondary MRFs, myogenin and MRF4, and are marked by the expression of proteins such as myosin heavy chain. Myogenin-null mice contain normal myoblasts, but are devoid of myofibers, as differentiation never occurs in these cells (Nabeshima et al., 1993). In contrast, the
absence of MRF4 results in a four-fold increase in myogenin expression and normal muscle development (Patapoutian et al., 1995). Deletions of many of these genes lead to delays during development. A subset of satellite cells was found to express myogenin before dividing, or perhaps without dividing (Cornelison and Wold, 1997).

Myf5 may precede MyoD in this commitment pathway (Hirsinger et al., 2001). Myf5 expression in developing embryos is detected initially, coexpressed with Pax3 (Ott et al., 1991). Cornelison and Wold (1997) found an expression of Myf5, MyoD, or a combination after satellite cell activation. It is possible that the dual expression of these two MRFs occurs as an initial coexpression or that it represents a secondary state in expression. Most cells would ultimately express the combination along with myogenin, and sometimes all four MRFs simultaneously. Expression of myogenin was exclusive to cells expressing MyoD and Myf5. Cornelison and Wold (1997) also determined that MRF4 expression was limited to cells also containing the other three MRFs.

Pax7

Pax7, the most reliable protein marker of satellite cells functions as a nuclear paired-box transcription factor in satellite cells. Required for satellite cell function, Pax7 contributes anti-apoptotic properties to this population (Relaix et al., 2006). Mutant Pax7 knockout mice appear normal at birth, but survive only two to three weeks (Seale et al., 2000). These mice demonstrate a decrease in fiber size but not number, congruent with the inability to repair damaged fibers. Mutant mice that survived to six months of age experienced severe muscle wasting and characteristic curvature of the spine due to a lack of satellite cells (Kuang et al., 2006). This appears to be due to a loss of satellite cells to apoptosis, or cell death, rather than a failure of the lineage to develop (Relaix et al., 2005).
The interplay between Pax and MRF genes provides a likely scenario for the self-renewal capacity of satellite cells. Pax7 and its parologue Pax3 control MyoD activation. The myogenic regulatory factor MyoD plays an essential role in commitment of cells down a myogenic pathway (Relaix et al., 2006). The most naïve satellite cells contain Pax7 and do not express MyoD. This population remains in an inactive state to function in self renewal. However, Pax7 does not promote quiescence (Zammit et al., 2006). Activation of satellite cells up-regulates production of MyoD, demonstrated by protein and mRNA quantities (Zammit et al., 2002). MyoD/Pax7+ cells proliferate.

Pax7 protein can drive transcription in quiescent, activated, and proliferating satellite cells and may help to promote proliferation (Zammit et al., 2006). Injury sites possess higher levels of MyoD, leading to differentiation (Chen and Goldhamer, 2003). Upon adoption of a myogenic lineage, these myoblasts express MyoD but not Pax7. Pax7 expression prevents myogenin, indicative of a committed myoblast (Olguin and Olwin, 2004). Terminally differentiated myotubes express myogenin and myosin heavy chain and lose their Pax7 identity.

Pax3

Pax3 proves critical for the delamination and migration of muscle progenitor cells from the somite to the limb buds. Kassar-Duchossoy et al. (2005) report Pax3 origins in the dermomyotome of the somites. Mutants for Pax3 lack limb muscles. Pax3 knockout mice cannot survive past mid-gestation, 14.5 days after conception (Bober et al., 1994). Montarras et al. (2005) noted a down-regulation of Pax3 before birth.

Relaix et al. (2006) found Pax3 abundant in mouse diaphragm muscles and sparse in hindlimb muscles. This group identified Pax3 in the forelimbs and trunk but not in the head or rib area. Relaix et al. (2006) further describe a clear heterogeneity of satellite cell populations expressing Pax3 and Pax7 or Pax7 alone. Deletion of Pax3 results in an absence of these
muscles (Goulding et al., 1994). Kuang et al. (2006) speculate on the role of Pax3 in the adult muscle fibers, including formation of muscle spindles, neuromuscular junctions, myotendinous attachments, or other muscle specializations.

Pax3 has unique and common functions to its homologue Pax7 in satellite cells. Most non-differentiating, quiescent satellite cells express both Pax3 and Pax7 (Relaix et al., 2006), representing the most naïve muscle progenitor cell. Pax3/Pax7+ cells without muscle markers reside in skeletal muscle (Kassar-Duchossoy et al., 2005). Pax3 exists in quiescent and activated satellite cells. Conboy and Rando (2002) report transient expression of Pax3 during activation. However, satellite cells do not require Pax3 while entering myogenesis and down-regulate it over time (Kassar-Duchossoy et al., 2005). Research suggests unique roles for each protein, with Pax3 promoting proliferation of muscle precursors (Relaix et al., 2004).

In embryological development, muscle progenitor cells are not present in the absence of Pax3, with Pax3 necessary for the development of the hypaxial dermomyotome. Pax7 is not expressed in these cells. In these experiments, Pax7 can replace most of the functions of Pax3 (Relaix et al., 2004). However, Pax3 is not capable of rescuing Pax7 functions in postnatal development of skeletal muscle. Relaix et al. (2006) performed research in this area with the use of a Pax7LacZ/LacZ mutant mouse. At postnatal day 3 (P3), Western blots identified the presence of Pax3 at a reduced level in the satellite cells. By P10, satellite cell presence is reduced to 10% of normal levels. Myonuclei are also affected, reduced by 50%, and muscle fibers are smaller in size. At day 11, 83% of satellite cells are absent in both muscles that contain Pax3, and those that do not.

Relaix et al. (2006) analyzed the affect of Pax7 absence of the proliferation of satellite cells. Primary cultures of satellite cells from Pax7LacZ/LacZ and Pax7LacZ/+ mice were monitored.
In each myogenic colony, cells were reduced by 25-30% in muscles containing and void of Pax3 expression. Relaix et al. (2006) also determined that this reduction was not based on withdrawal from the cell cycle. Therefore, Pax3 cannot compensate for the antiapoptotic properties of Pax7 (Relaix et al., 2006).

**Notch**

The Notch signaling pathway functions in regulation of satellite cell proliferation (Chen and Goldhammer, 2003). Activation of the Notch pathway also inhibits myogenic differentiation, as Notch blocks MyoD activation in vertebrates (Kopan et al., 1994; Nofziger et al., 1999; Shwaber et al., 1996). This rapid proliferation and inhibition of differentiation promotes a rapid expansion of a pre-myoblast population from a satellite cell.

The N or Notch receptor exists as a heterodimer in the plasma membrane. This type 1 transmembrane protein consists of an ectodomain, or Notch extracellular domain (NECD) and a membrane-tethered Notch intracellular domain (NICD) (Le Borgne et al., 2005). Modifications to Notch initiate the canonical Notch pathway (Kopan, 2002).

Injury activates this pathway and stimulates production of the Notch ligand, Delta, within 24 hours. Delta can be found on the membranes of activated satellite cells and adjacent muscle tissue (Conboy and Rando, 2002). The NECD binds Delta from another cell resulting in two proteolytic cleavages. Extracellular proteases mediate cleavage at the S2 site, resulting in an activated, membrane-bound form of Notch. Further processing by the γ-secretase complex at the S3 site initiates a release of the NICD into the cytoplasm. The NICD localizes to the nucleus and associates with a DNA binding protein, CSL. Active Notch allows CSL to activate target gene transcription. In the absence of Notch signaling, this transcription is repressed due to CSL binding to a corepressor complex. (Le Borgne et al., 2005).
This evolutionarily conserved pathway functions in tissue morphogenesis during development and tissue maintenance and repair in the adult. In vertebrate neurogenesis, Notch activation inhibits differentiation by preventing exit from the cell cycle. Progression of satellite cells to become fusion competent myoblasts depends on Notch signaling (Conboy and Rando, 2002).

Delta expression in early myogenesis causes a strong down-regulation of MyoD, leaving the early myogenic marker genes Pax3 and Myf5 genes unaffected. This over-expression of Delta results in an absence of differentiated muscles (Hirsinger et al., 2001). Neural progenitor cells cannot exit cell cycle when exposed to an over-expression of Delta (Henrique et al., 1997). However, exit from the cell cycle in myogenic cells is not blocked, as formation of the myotome is not prevented. Therefore, muscle differentiation in postmitotic myogenic cells depends on Notch signaling with down-regulation of Notch leading to terminal differentiation (Hirsinger et al., 2001).

**Numb**

Numb, a conserved cell membrane-associated protein, binds to the NICD to antagonize the Notch signaling pathway (Guo et al., 1996; Santolini et al., 2000). Numb interacts with Notch through its N-terminal phosphotyrosine binding domain in two regions (Guo et al., 1996). Increasing with satellite cell activation, Numb promotes cell commitment and progression down a myogenic lineage, ceasing the population expansion caused by Notch (Conboy and Rando, 2002). Numb-mediated cell fate determination depends on the ability to inhibit Notch (Bhalerao et al., 2005).

First identified in *Drosophila*, this protein also controls cell fate in the central and peripheral nervous system development (Uemura et al., 1989). Numb knockout mice die as early embryos due to a failure of neural tube closure, as Numb proves essential for *Drosophila* in cell

Numb inhibits Notch by targeting this protein for degradation via endocytosis or ubiquitin-mediated protein degradation. The endocytosis theory claims that Notch is internalized by vesicles and degraded. This alters the number of receptors on the cell surface (Le Borgne et al., 2005). Alternatively, other studies focus on ubiquitination of Notch. This multi-step reaction covalently bonds the NICD with ubiquitin, a small polypeptide. This process targets Notch for proteasome degradation and makes it less efficient as a transactivator. Mammalian cells utilize this pathway (Oberg et al., 2001; Wu et al., 2001). Guo et al. (1996) reported that Numb prevents nuclear translocation of Notch.

Satellite cell progeny assume fate through Numb-mediated asymmetric cell division (Conboy and Rando, 2002). In asymmetric cell division, Numb concentrates and overlays the spindle poles, ultimately segregating to one daughter cell which differentiates, while the other cell does not and remains as a progenitor cell (Shen et al., 2002; Johnson, 2003). Immunocytochemistry revealed Numb segregating in a crescent shape with this adoption of divergent fates. Adoption of Numb leads to the co-expression of committed myogenic proteins, such as Pax7, which also participates in asymmetric cell division (Conboy and Rando, 2002).

Experiments by Bhalerao et al. (2005) have shown that different classes of asymmetric cell division exist in *Drosophila*. For example, in dividing ganglion mother cells and during heart development, Numb function can persist independent of its ability to segregate asymmetrically. Some functions of Numb remain independent of cell division; the protein can influence cell lineage when mitosis is inhibited (Han and Bodmer, 2003).
Asymmetric Cell Division and Self-Renewal

Satellite cells may maintain their Pax7 identity through asymmetric cell division. This conserved method partitions genetic information during mitosis. During cell division, two daughter cells are produced. Normally these cells each receive an identical genome. Asymmetric cell division creates a difference in the inherited information and ultimately cell fate (Roegiers and Jan, 2004). Dividing satellite cells may distribute Pax7 in this manner. One daughter cell receives Pax7 and returns to a dormant state, replenishing the satellite cell compartment. The other Pax7-null daughter expresses myogenic regulatory factors and differentiates, fusing to myofibers (Charge and Rudnicki, 2004). Numb, a negative regulator of Notch, may affect Pax7 asymmetric division.

An alternative model, the ‘stochastic’ model presents an initial symmetric proliferation period for satellite cells. After this period, one or more progeny will return to quiescence. The remaining progeny differentiate and fuse to the muscle fiber, resulting in growth and repair (Dhawan and Rando, 2005). Work with isolated satellite cells by Zammit et al. (2004) reinforces the stochastic model. In culture, satellite cells initially proliferate, expressing Pax7 and MyoD. After 72 hours in culture, a significant portion exists that is Pax7 positive and MyoD negative. It is likely that satellite cell self-renewal functions as a combination of different forms of division.

The Numb protein, which also participates in asymmetric cell division, segregates selectively, suggesting a role of numb in self-renewal. Current findings show Numb segregation two days before the appearance of differentiation markers, insinuating that Numb carries this role of self-renewal rather than cell fate determination. In this study by Shinin et al. (2006), Numb cosegregated with the more intensely stained, or mother, centrosome. This group also found evidence of template strand cosegregation in satellite cell derived cultures through BrdU labeling, which creates an immortal strand to protect the genome from DNA replication errors.
Ninety percent of cells were found to cosegregate template DNA and Numb asymmetrically, however Numb asymmetry probably does not facilitate cosegregation of template DNA strands. Pax7 was expressed in doublets colocalizing template DNA. In fact, the immortal DNA strands were found together with Pax7 and Numb, and these cells did not differentiate.

**Cell Cycle and Quiescence**

Five sections partition the cell cycle into the following phases: G0, G1, S, G2, and M. G0 indicates the quiescent state in which satellite cells reside in adult muscle. This resting state occurs before cells divide or differentiate. Quiescent cells maintain basic metabolic functions but do not replicate. They exist outside of the cell cycle. Low growth factors or nutrients stimulate entry into G0. Cells may then remain in this non-dividing state or differentiate. Gap 1 (G1) describes a period where the cell is preparing to reproduce its DNA. In the G1 phase, myogenic cells contain higher expression levels of MyoD (Kitzmann et al., 1998). During S, the DNA is duplicated, creating a genome for each daughter cell. The cell begins division in Gap2 (G2) and divides during the mitosis (M) phase. Cells exiting M feed back into G1, were they may continue the cycle or exit into G0. Cyclin expression marks the stages of the cell cycle.

The reversible quiescence demonstrated by satellite cells provides them a unique niche in the commitment pathway. Cells that do not participate in self-renewal exit the cell cycle to differentiate and cannot reenter. Satellite cells retain the ability to enter and withdraw from the cell cycle multiple times. Research focuses on this quality as many questions remain unanswered.

**Pocket Proteins**

Mammalian cells express three retinoblastoma tumor suppressor (pRB) family members: pRB, p130, and p107. These pocket proteins function in regulation of cell cycle progression. In their hypophosphorylated state, pocket proteins are able to bind to the E2F transcription factors
to inhibit transcription. Phosphorylation via cyclin/CDK activity releases pocket proteins from their E2F complexes, permitting transcription (Dyson, 1998). Though these pocket proteins are closely related, they have different affinities for the E2F proteins, resulting in distinct, but overlapping function in regulation of the cell cycle (Moberg et al., 1996). pRB has been shown to regulate MyoD in muscle (Yee et al., 1998).

Balciunaite et al. (2005) worked to define functional differences between the highly related and less researched members of the pRB family, p130 and p107. T98G cells were synchronized in hydroxyurea with a 12 to 16 hour block, followed by release, allowing for homogeneity within the cell populations. Western blot analysis revealed p130, but not p107 or pRB presence in cells at G0. Continuously cycling cells were shown to express all three of the pocket proteins. Balciuniaite et al. (2005) deducted that occupancy of E2F-regulated promoters by transcriptional repressors is not the same for quiescent and cycling cells.

To identify targets for E2F4 and pocket protein binding during early G1 in cycling cells, Balciuniaite et al. (2005) performed genome-wide location analysis by use of a custom DNA microarray with human gene promoter regions. E2F4, p130, and p107 occupied 301, 226, and 244 gene promoter elements, respectively. Comparison revealed that 171 of these targets were bound by all three of these factors. The remaining sites that showed binding were therefore unique to E2F4. E2F/ p107/ p130 complexes bound to genes with a variety of functions, including apoptosis, mitochondrial biogenesis, transcriptional regulation, protein ubiquitination, and organelle assembly and function. Functions unique to E2F4 and/or p107 promoters include stress response, signal transduction, and immune response. A subset of E2F4-bound genes are not dependent on the pocket proteins, as they functioned normally in a triple-knockout. This
work identified unique combinations of E2F4, p130, and p107 that exhibit distinct histone acetylation signatures.

**E2F Family**

Members of the E2F gene family include E2F1, E2F2, E2F3, E2F4, E2F5, and E2F6. These transcription factors, with the exception of E2F6, are implicated in the control of genes essential for cell cycle progression, including *cyclins A* and *E* (Botz et al., 1996). pRB family binding mediates the effects of these proteins. Cell cycle-dependent fluctuations in the pocket proteins and the E2Fs also influence regulation of E2F activity. The localizations of the pocket proteins may interact with the myogenic factors, together promoting differentiation (Moberg et al., 1996). During embryogenesis, levels of E2F, pRB, and cyclin fluctuate greatly (Jiang et al., 1997). Changes in expression pattern and activity of these factors are essential for differentiation of myoblasts into multinucleated myotubes (Shin et al., 1995).

The E2F family, along with the pocket proteins is able to repress transcription through the recruitment of histone modifying enzymes to target promoters. Action of histone deacetylases leads to histone H3 and H4 deacetylation and transcriptional silencing. Histones associated with E2F regulated promoters are acetylated through the cell cycle in proliferating cells. However, acetylation displays a decrease in early G1. Histone acetylation profiles for the E2F genes differ from promoter to promoter. Promoters are generally acetylated in the S, G2, and M phases of the cell cycle (Balcuinaite et al., 2005).

In 1995, Itoh et al. introduced E2F5 into the E2F family through means of cloning, structural characterization, and tissue expression pattern. Analysis provided that *E2F5* is highly conserved between the human and rat. However, E2F5 shows considerable variation from E2F1, E2F2, or E2F3. E2F4 and E2F5 are thought to occupy a separate branch from the previously mentioned proteins. E2F5 mRNA distribution among adult rat tissues displayed differences
from E2F1. These variations also manifested in cell cycle expression pattern of vascular, smooth muscle cells. The variation between the two branches of the E2F family insinuates participation in different regulatory networks. E2F1, E2F2, and E2F3 have been termed transcriptional activators, interacting primarily with pRB. E2F4 and E2F5 are transcriptional repressors and preferentially bind to p107 and p130 (Gaubatz et al., 2000).

In cycling cells, E2F4 and E2F5 display a primarily cytoplasmic localization, due to the lack of an NH₂-terminal nuclear localization signal (NLS) (Lindeman et al., 1997). However an alternatively spliced version of the heterodimerization protein DP-3, a binding factor that encodes the NLS, could allow a nuclear localization of E2F4 (Puri et al., 1998). E2F4 and E2F5 depend on the members of the DP, or pRB families for their nuclear translocation (Lindeman et al., 1997; Puri et al., 1998). Simultaneous inactivation of E2F4 and E2F5 in mice induces neonatal lethality, implying overlapping function for these two proteins (Gaubatz et al., 2000).

E2F1, E2F2, and E2F3 possess the NLS (Lindeman et al., 1997). Nuclear E2F1 or E2F4 of undifferentiated myoblasts promotes progression into S phase and blocks cell cycle exit, even with the removal of mitogenic signals. During terminal differentiation, active E2F blocks exit of cell cycle (Puri et al., 1998).

Gill and Hamel (2000) investigated localization of pRB and E2F family members in Rat-1 fibroblasts and L6 myoblasts during reversible growth arrest through immunofluorescence. E2F expression proved nuclear in arrested cells and cytoplasmic in S phase cells. Results demonstrated that the presence of very weak mitogenic signals (0.2% serum) could induce a variation of compartmentalization of the E2F and pRB proteins.

Subcellular localization of these signals were then investigated in differentiated, multinucleated L6 myotubes in an irreversible quiescent state. After five days of differentiation,
the pocket proteins could be detected in the nuclei of the myotubes and undifferentiated
myoblasts. Weak cytoplasmic staining was observed. Western blots of these fractions reiterated
this conclusion. Specific anti-E2F antibodies were employed to detect localization. E2F2 was
expressed in a primarily nuclear pattern, with slight cytoplasmic staining. E2F4 was detected in
the nuclei and cytoplasm of these cells. E2F1, E2F3, and E2F5 exhibited a solely cytoplasmic
staining pattern, with no nuclear fluorescence. The observed localizations are thought to occur
through an active transport mechanism. E2F1 and E2F5 results proved incongruous with
proliferating myoblast data.

*In vivo* evidence was obtained by this group via immunohistochemical analysis of day
E18.5 murine embryo sections. Skeletal muscle adjacent to developing ribs exhibited
cytoplasmic E2F4, with nuclei devoid of signal. E2F1 also revealed a cytoplasmic staining
pattern.

Gill and Hamel (2000) also examined the effects of significant increases to nuclear
localization of E2F1 and E2F4. This was achieved through the use of adenoviruses expressing
green fluorescent protein (GFP) fused to E2F1 or E2F4. Infection at 5x10^9 pfu in multinucleated
myotubes demonstrated cytoplasmic E2F1 with E2F4 localizing to the nuclei and the cytoplasm.
Cytoplasmic localizations of the E2Fs in terminally differentiated myotubes infers a mechanism
for the halt of cell cycling and DNA synthesis for the differentiated cell. An override of
cytoplasmic sequestering in this system induced nuclear localizations of these proteins, as
confirmed by Western blot. The nuclear localization caused these terminally differentiated
myotubes to enter S phase, mediated by altered expression of cell cycle regulatory factors. E2F4
induced expression of the positive cell cycle regulatory factors *cyclin A, cyclin E*, and *cdk2.*
Data by Gill and Hamel (2000) suggests that the terminally differentiated myotube nuclei are competent to reenter the cell cycle upon this E2F-mediated proliferation signal. Cytoplasmic localization of E2F thus plays an essential role in the maintenance of the postmitotic state of myotube nuclei.

**LEK1**

Centromere protein-F (CENP-F) and a family of proteins including mitosin, CMF1, and LEK1 are implicated in cell division in different species. CENP-F, a 367kDa human protein, was originally derived from a patient with autoimmune disease (Rattner et al., 1993). This immunoreactive protein was cloned from a HeLa cDNA library. During the mitosis of a dividing HeLa cell, CENP-F will localize to the outer plate of the kinetochore. CENP-F is up-regulated during late S phase, and localizes to paired foci near the centromere of each chromosome during prophase and metaphase. During anaphase, this protein can be found in the intracellular bridge and ultimately creates a narrow band on either side of the midbody during telophase. Rapid turnover of CENP-F during mitosis can be detected by pulse chase experiments (Liao et al., 1995).

Nearly identical in sequence to CENP-F, mitosin also behaves similarly in the human. This nuclear protein binds to the tumor suppressor protein retinoblastoma. In dividing tissue culture cells, mitosin localizes to the outer plate of the kinetochore, and also binds microtubules. During mitosis, mitosin localizes almost identically to CENP-F (Zhu et al., 1995).

Chicken CMF1 shares 56% of the cDNA nucleotide sequence with these two human proteins, and exhibits a 65% similarity at the amino acid level. This protein plays a role in early chicken heart myogenesis, possibly functioning in differentiation of this cardiac muscle. Wei et al. (1996) infected cardiomyocytes with CMF1 antisense containing retrovirus. Disruption of the
CMF1 RNA function led to a loss of stage appropriate markers of differentiation in these cells. CMF1 also has been detected in skeletal muscle.

Goodwin et al. (1998) reported a novel gene product- LEK1, cloned from murine cDNA. This protein shares structural elements with CENP-F, mitosin, and CMF1. Homology exists at the primary, secondary, and tertiary levels. Like CMF1, CENP-F, and mitosin, LEK1 structure reveals $\alpha$-helices, separated by turns and a proline-rich, globular C-terminus. LEK1 also contains perfect leucine zippers, important for protein interactions with DNA or other proteins. LEK1 cDNA is 75% identical to that of CENP-F and mitosin. These proteins contain a characteristic 40% of the amino acids leucine (L), glutamic acid (E), and lysine (K), defining these three proteins as the LEK family of genes, including murine LEK1. Post-translational modification includes a cleavage that produces a longer NH$_2$-terminal peptide and a shorter COOH-terminal peptide of LEK1. This shorter portion participates in a nuclear localization.

Goodwin et al. (1998) explored LEK1 mRNA expression during mouse development with Northern blot experiments. The early embryo demonstrated the highest levels of expression. The head and caudal regions posterior to the heart expressed LEK1 message at 9.5dpc; the whole embryo expressed message at 8.5dpc. Relative levels also seemed to increase during the heart and liver development. Northern blots revealed high levels of expression in 14dpc murine hearts until 4 days postpartum. The head and brain experienced down-regulation of transcript with age. Ubiquitous expression during murine development ultimately resulted in a down-regulation after cessation of mitosis, with little expression found in the 7-day-old mouse. A low, but detectable level of LEK1 expression was observed in adult heart muscle. *In vivo* expression of this protein hints at a unique role for LEK1 in mitosis and differentiation.
In vitro work by the same group utilized a LEK1 antibody to determine localization. C2C12 murine myoblasts demonstrated nuclear localization of LEK1 in over 95% of cells. Some weak, cytoplasmic staining was also noted. Mitotic cells expressed LEK1 protein in all parts of the cell during prophase. Metaphase and anaphase in these cells yielded fully cytoplasmic LEK1 with staining absent in the presence of condensed chromosomes. Differentiation of these cells into myotubes reveals LEK1-positive nuclei and increased cytoplasmic staining. Terminal differentiation and withdrawal from the cell cycle revealed a down-regulation of LEK1.

Ashe et al. (2004) proposed a theory that LEK1 functions as a regulator of pocket protein activity. This work indicates that LEK1 interacts with all three pocket proteins: p107, p130, and pRB. This association provides the possibility for LEK1 to affect activity in all members of the Rb family. The “pocket domain” of pRB that is involved in Rb association with other targets specifically interacts with the LEK1 Rb-binding motif. LEK1 is able to recognize sequences in all three pocket subdomains of Rb in vivo. Other proteins that are known to associate with the pocket domains include the E2Fs, which recognize and bind to sequences in each subdomain in a manner similar to LEK1 (Chellappan et al., 1991).

Disruption of LEK1 protein translation from mRNA results in a decrease in cell proliferation, disruption of cell cycle, and apoptosis, or cell death. This disruption was achieved utilizing morpholino antisense oligomers, which exhibit a higher specificity and are more stable than general antisense oligomers. LEK1-depleted cells demonstrated an arrest at the G1/S checkpoint in the cell cycle. These cells were fewer in number than control cells. Also, a delay in cell cycle progression caused depleted cells to fall one cell cycle behind control cells. Cells treated for a duration of three days showed over 50% of cells undergoing apoptosis, not observed.
in control populations. Depletion of LEK1 yielded results similar to the up-regulation of pocket proteins (Ashe et al., 2004).

This role of LEK1, along with its role in developing cells, suggests a mechanism for LEK1 action. Ashe et al. (2004) propose that LEK1 functions similarly to phosphorylation, disrupting the association of Rb with its necessary binding partners. LEK1 binding to Rb will maintain cells in a proliferative, non-differentiating state.

The role of the larger, uncleaved protein cytoplasmic LEK1, termed cytLEK1, was investigated by Soukoulis et al. (2005). Observations of 3T3 fibroblasts and C2C12 myoblasts through immunocytochemical analysis revealed a cytoplasmic distribution pattern for cytLEK1. This protein also localizes more intensely in the perinuclear region, excluded from regions of the nucleus containing DNA.

A yeast two-hybrid screen determined that cytLEK1 interacts with nuclear distribution gene E (NudE) in a conserved pathway that regulates the function of dynenin and microtubules. The binding domain identification for each molecule, along with coimmunoprecipitation and colocalization techniques revealed a specificity for the binding of these two proteins. Confocal deconvolution analysis demonstrated a colocalization of cytLEK1 with endogenous NudE and its binding partners, LIS1 and dyenin. Disruption of cytLEK1 function through mopholino antisense oligomer treatment resulted in alteration of microtubule organization and cellular shape. The microtubule network aggregated around the nucleus and resulted in a rounded cell shape. These results are consistent with a defect in the LIS1 pathway, showing an essential role for cytLEK1 in this pathway (Soukoulis et al., 2005).

CytLEK1 is proposed to regulate plasma membrane recycling by Pooley et al. (2006). This research group utilized similar methods as Soukoulis et al. (2005) to identify interactions
and binding domains. A novel interaction and high degree of colocalization was discovered between cytLEK1 and SNAP-25, a component involved in membrane docking and fusion. This protein pair colocalizes and coprecipitates with various components of the plasma membrane recycling pathway: Rab11a, myosin Vb, VAMP2, and syntaxin 4. Depletion of cytLEK1 through morpholino antisense oligomer treatment resulted in altered trafficking. This study indicates that cytLEK1 serves as a link between the microtubule network and recycling vesicles through association with SNAP-25, possibly performing a key role in regulation of the recycling endosome pathway.

Reed et al. (2007) used a proteomic approach to assess possible interaction of LEK1 and E2F5 in myoblast quiescence. Inducible-Raf myoblasts in cell culture can block cell cycle progression through MEK/ERK activity in a reversible manner through elevated levels of Raf. Nuclear extracts from Raf-induced and vehicle control populations of this cell line were separated via SDS polyacrylamide gel electrophoresis and visualized by silver staining. Two protein spots were identified to be unique to the Raf-induced cells only: E2F5 and LEK1. Immunofluorescent detection of these proteins verified their localizations.

Control myoblasts demonstrated E2F5 in an exclusively cytoplasmic localization. Upon induction of elevated levels of Raf, this protein translocated to the nucleus in 70% of the myoblasts. LEK1 behaved in a similar manner. LEK1 resides in both the nucleus and cytoplasm of growth-arrested, vehicle control cells. However, a 15 minute induction of sustained Raf signaling allowed a nuclear translocation of protein. Immunodetection of a small remainder of cytoplasmic LEK1 was possible. The brief time necessary to elicit a response in this model system provides the possibility that nuclear expression of LEK1 and E2F5 demarcate myoblast entry into quiescence.
Analysis of the distribution of pocket proteins was observed. p130 and p107 showed no changes between control and treated cells. However, Raf-induced myoblasts experienced a nuclear translocation of pRB, with residual cytoplasmic immunoreactivity. Less than 20% of Raf arrested cells retained exclusive cytoplasmic localization of pRB. The nuclear translocation of these three proteins in Raf-arrested cells was shown to be dependent on the MEK/ERK signaling cascade. Treatment with a MEK inhibitor abolished the nuclear translocation. LEK1 contains an Rb-binding motif, creating the possibility that LEK1 serves as a shuttle for pRb localization to appropriate parts of the chromosome. Though p130 was not detected nuclearily at early timepoints, nuclear accumulation of this protein may serve a function in long term maintenance of the quiescent state.

Reed et al. (2007) substantiated the culture system with immunofluorescent staining in isolated mouse satellite cells. After 24 hours in primary culture, few PCNA positive cells existed, verifying the quiescent state of these satellite cells. Eighty percent of the cells expressed Pax7. Nuclear E2F5 and LEK1 described 82% and 77% of the cells, respectively. Full activation of the satellite cells, as determined by morphological and protein expression changes, demonstrated E2F5 and LEK1 expression primarily in the cytoplasm, with faint nuclear localization.

**Myogenesis Inhibition by Activated Raf**

The Ras oncogene family is comprised of 21Kd proteins on the cytoplasmic side of the cell membrane and consists of 3 members: Ha, K, and N (Lowy and Willumsen, 1993). The Ras pathway, once activated, can induce a different phenotype of small, mononuclear, spindle-shaped cells that is differentiation defective in 23A2 murine embryonic skeletal myoblasts. This state is achieved by blocking myogenesis and is marked by an inhibition of MyoD (Konieczny et al., 1989). This activation induces a phosphorylation cascade involving GDP and GTP (Burgering
Ras signaling influences the transformation of fibroblasts, among other biological responses (Satoh et al., 1992). Interestingly, this state is reversible, indicating that signaling pathways must be maintained at a certain level to promote the effect (Nakafuku, 1992).

The Ras gene acts upstream of Raf, and ultimately, MEK1 and the members of the mitogen activated protein kinase (MAP kinase) family (Moodie and Wolfman, 1994). 23A2 myoblasts transformed to express oncogenic Ras (Ras9 cells) display increased levels of MAP kinase activity in their differentiation-defective state, whether cultures were maintained in growth or differentiation media (Weyman et al., 1996). Proliferation of Ras9 cells with the addition of a MEK1 inhibitor, PD 098059, proves that Raf acts along this pathway (Alessi et al., 1995). Ras9 cells in this induced, differentiation-defective state could be considered quiescent, or in the G0 phase of the cell cycle.

Weyman et al. (1996) have shown that MAP kinase is not necessary to achieve this differentiation defective phenotype, while the altered morphology is a result of MAP kinase. Previous work has shown that protein kinase C is required to initiate the differentiation defective state, while it is not needed for the maintenance of this state (Vaidya et al., 1991). Dorman and Johnson (2000) demonstrated that Raf inhibition of muscle differentiation does not depend on Activator Protein 1 (AP-1). This inhibition does not result from changes in function of the MRFs (Kong et al., 1995).

Raf over-expression in myoblasts and mature myocytes inhibits skeletal myogenesis. Embryonic chick myoblasts over-expressing a constitutively active Raf were not able to form multinucleated myofibers. Residual myofibers present had an atrophic phenotype. This morphology was not due to entry into the cell cycle or apoptosis (Dorman and Johnson, 1999).
Dorman and Johnson (2000) demonstrated that a strong Raf signal in myoblasts does not induce terminal differentiation, or an irreversible senescent state. Myoblasts released from Raf arrest are able to reenter the cell cycle and proliferate. Reed et al. (2007) demonstrated that inducible-Raf myoblasts provide a model system to analyze cell cycle and quiescence. With a 48 hour induction of elevated levels of Raf, the myoblast population failed to increase in number. Control myoblasts doubled in number during this two-day period. Acidic galactidose staining revealed that no treated cells died or became senescent. Removal of stimulus allowed cells to reenter the cell cycle, proving that this form of growth arrest is reversible. No negative effects on mitosis occurred. Terminal differentiation is not induced with induction of elevated levels of Raf, unlike with serum deprivation, as shown by a lack of myogenin expression.

**Satellite Cell Activation**

Once activated by injury or stimulus for muscle hypertrophy, such as weight bearing exercise, stretch, or trauma, satellite cells exit the quiescent state and proliferate, forming myogenic precursor cells (mpcs). These mpcs undergo several rounds of division and eventually differentiate and fuse to muscle fiber, resulting in postnatal muscle growth and repair as well as the formation of the adult muscle mass (Bischoff and Heintz, 1994). Damage at one end of a muscle fiber will activate satellite cells along this fiber, promoting proliferation and recruiting migration to the injury site (Schultz et al., 1985). Satellite cell isolation inevitably leads to activation, making *in vitro* analysis difficult (Zammit, 2004). The regenerative capacity of these cells is so great, that less than ten engrafted satellite cells can contribute to over 100 myofibers (Collins et al., 2005).

Myostatin or GDF8, a member of the TGF-β superfamily of proteins, is a potent negative regulator of satellite cell activation by inhibition of cell cycle progression and signals satellite
cell quiescence (McCroskery et al., 2003). Satellite cells released from myostatin regulation in a 
myostatin knockout mouse enables a significant increase in muscle regeneration and growth 
(McPherron et al., 1997). McCroskery et al. (2003) noted an up-regulation of myostatin mRNA 
in Pax7 positive cells. Analysis of cell cycle proteins revealed myostatin regulation of the cell 
cycle at the G₁ to S transition. This secreted growth factor maintains satellite cell quiescence.

**Hepatocyte Growth Factor**

Hepatocyte growth factor (HGF) to date, is the only growth factor found to be capable of 
activating quiescent satellite cells. Allen et al. (1995) elucidated the role of HGF by utilizing 
quiescent satellite cells isolated from nine-month-old rat skeletal muscle. These cells from aged 
animals showed a lag phase before their first division when compared to neonatal satellite cells. 
Satellite cells from the aged mice divided 48-60 hours post plating. HGF increased proliferation 
in a dose dependent manner prior to 48 hours. Treated satellite cells also expressed proliferating 
cell nuclear antigen (PCNA) earlier than controls. Earlier expression of this S phase marker 
insinuates earlier entry into the cell cycle. Tatsumi et al. replicated this work *in vivo* in 1998 
using BrdU incorporation to mark dividing cells after HGF treatment.

Crushed muscle extract (CME) contains HGF and also activates satellite cells. The HGF 
in the CME is released upon injury in skeletal muscle and causes the activation of satellite cells 
described in this *in vivo* work. Tatsumi and Allen (2004) demonstrated that HGF resides 
tethered to the skeletal muscle extracellular matrix in its active, proteolytically cleaved form as a 
disulfide-linked heterodimer of 60kDa alpha-chain and a 30kDa beta-chain.

HGF is not only released from skeletal muscle upon injury, but also by satellite cells. 
Sheehan et al. (2000) demonstrated that cultured satellite cells from 12-month-old rats synthesize 
and secrete HGF, and that this HGF can behave in an autocrine fashion. Reverse transcription-
polymerase chain reaction (RT-PCR) revealed HGF mRNA presence in cultured satellite cells at
12 hours post-plating. Message was detectable early in culture, increasing between 36 and 48 hours. Neutralizing HGF antibodies added during the proliferation phase (42-90 hours) reduced cell proliferation.

In fact, activation of a satellite cell is defined by the binding of HGF to its receptor, c-met and can be determined by satellite cell expression of Myf5 or MyoD (Tatsumi et al., 1998). While c-met is not exclusive to satellite cells, it marks quiescent and activated satellite cells (Cornelison and Wold, 1997). Binding of HGF to its receptor elicits multiple signaling cascades including Mitogen-activated protein kinase (MAPK) and phosphatidyl inositol 3 kinase pathways. MAPK activation leads to subsequent binding of activating protein-1 (AP-1) to DNA. Action through these pathways leads to transcription of genes for growth and cell division (Furge et al., 2000). HGF also increases migration of muscle cells (Bischoff, 1997).

No other growth factor has been shown to induce satellite cell activation from a quiescent state. However, fibroblast growth factors (FGFs), platelet-derived growth factor, and insulin-like growth factor are each capable of promoting proliferation in satellite cells that have been previously activated (Johnson and Allen, 1995). Fibroblast growth factors such as FGF-2 and FGF-6 specifically promote proliferation in vitro, while they down-regulate the ability of these cells to differentiate. Scatchard analysis revealed high affinity binding receptors for FGF-2 in young and adult isolated satellite cells. Insulin-like growth factor encourages cells to proliferate, as well as differentiate (Husmann et al., 1996).

Nitric Oxide

Nitric Oxide (NO) is a small, easily diffusible molecule expressed ubiquitously throughout the body. In the muscle, high levels of NO are produced constitutively by neuronal nitric oxide synthase (NOS-Iµ), an isoform specific to skeletal muscle (Nakane et al., 1993). NOS-Iµ synthesizes NO as a product of the conversion of L-arginine to citrulline. The N terminus of
NOS-Iµ is complexed to α1-syntrophin, and ultimately linked to dystrophin in the cytoskeleton (Brenman et al., 1996).

Injury stimulates a release of NO from the muscle fibers, which is thought to mediate satellite cell activation. *In vivo* mouse muscle trials have utilized a non-specific NOS inhibitor, N-nitro, L-arginine methyl ester (L-NAME) prior to crush injury. Results included an inhibition of rapid satellite cell activation by NO. Satellite cell hypertrophy and binding of HGF to its receptor c-met did not occur. Satellite cell heterochromatin areas also remained condensed, revealed by electron microscopy. Muscle without inhibition yielded satellite cells that displayed organelle hypertrophy and HGF binding after just 10 minutes through a NO-dependent pathway. A less preferred pathway may exist, as satellite cell activation was delayed in these trials, and not inhibited completely (Anderson, 2000). Nitric oxide is also shown to mediate HGF release in mechanical stretch trials (Tatsumi et al., 2002). Smith et al. (2002) describe a role for NO in overload-induced hypertrophy and fiber type transition in skeletal muscle.

**Aging**

Bischoff (1994) reported that the greatest number of satellite cells exist in postnatal muscle. In the first few weeks after birth, the numbers of satellite cells in rodent hindlimb muscle declines from about 30% of total nuclei to less than 5% (Schultz and McCormick, 1994). As muscle matures, the number of satellite cells declines with age.

Along with a decrease in number of satellite cells in aged animals, other factors may contribute to the age-related impairment of satellite cell function. “Aging” of satellite cells may yield cells that are less responsive to stimuli. An alternative includes an age-related decline in signals that incite satellite cells to participate in repair. In a study by Conboy et al. (2003) the Notch signaling pathway was analyzed for possible involvement of this satellite cell activity with
Inhibition of Notch signaling with a Notch inhibitor in injured adult muscle led to poor muscle regeneration, characteristic of the response that is witnessed in aged muscle. Also, the number of Notch receptors is not changed with age. However, muscle fibers and satellite cells failed to effectively up-regulate Delta, the Notch ligand, in the case of injury. A “pseudo-ligand” was found to restore the regenerative potential to aged muscle, mimicking patterns observed in young animals. In terms of the Notch signaling pathway, it seems that the extrinsic cues depleted the regenerative potential of muscle regeneration, and that this loss of potential is reversible.

The results from this and other studies suggest that environmental cues, and not an age-related decline in satellite cell potential or function contribute to the decreased regenerative potential of aged satellite cells. Effective regeneration is impaired by a decreasing ability of these satellite cells to enter the cell cycle in vitro (Schultz and Lipton, 1982). However, normal proliferation results from these cells, despite the delay in division. Conboy et al. (2005) elucidated that serum from young mice restored effective muscle regeneration in aged muscles. Heterochronic transplantation studies by Carlson and Faulkner (1989) involved transplantation of minced muscle from young and old rat donors into young or old recipients with testing for regenerative potential. Results indicated that the host environment was more important than the age of the donor in determination of regeneration.

More recent studies involve parabiotically-paired mice which were surgically connected with a shared circulatory system. In this scenario, circulating factors from each mouse can affect the other. Pairings of young and old mice led to improved regeneration in the old mouse. With injury, increased expression of Notch and Delta influenced this system, along with an increase in satellite cell activation and generation of myoblasts. Pairings utilized young mice with green
fluorescent protein (GFP) tagged satellite cells. Negligible engraftment of satellite cells from young mice into old was noted, determining that either an exposure to the young environment, or cessation of exposure to the older environment, aided in more effective regeneration, and not contributions from the younger cells themselves. Young serum also was able to promote satellite cell activation in vitro (Conboy et al., 2005).

Verdijk et al. (2007) demonstrated a reduction in satellite cell number in type II skeletal muscle fibers of the elderly. Muscle biopsies from young and elderly healthy men were analyzed in serial cross sections for satellite cell content and muscle fiber type via an anti-Pax7 antibody and ATPase staining. Type I fibers reflected no differences between the young and elderly. However, type II fibers displayed a significant reduction in proportion and mean cross sectional area in elderly patients. Numbers of satellite cells per type II fiber were substantially reduced as well. This data was analyzed in terms of total fiber-associated nuclei and satellite cells per fiber area. Type II muscle fiber atrophy may play a key role in sarcopenia and the loss of skeletal muscle with aging.

**Supplementation**

Several studies have explored the possibilities of affecting satellite cell activity or number via supplementation. Johnson et al. (1998) focused on the affects of a trenbolone acetate (120mg) and estradiol (24mg) implant on satellite cells from yearling steers. Implanted steers demonstrated a 23% greater fed efficiency and 28% greater average daily gain than control steers. Satellite cells were isolated and cultured. Satellite cells isolated from implanted steers showed greater fusion percentage, yielding a greater number of myotube nuclei than satellite cells from control steers. \[^{3}H\]-thymidine incorporation rates were also greater for the satellite cells isolated from implanted steers 34 hours after plating. These results implicate that a trenbolone acetate and estradiol implant typically used in industry may result in activation of
satellite cell proliferation in vivo, and may play a role in the enhanced muscle growth witnessed with this supplement.

Sissom et al. (2006) indirectly evaluated the affects of melengestrol acetate (MGA) and progesterone (P4) on bovine muscle satellite cells. MGA supplementation at physiological and supraphysiological levels resulted in a decrease in [3H]-thymidine incorporation into DNA. P4 addition reflected similar results. mRNA levels of myogenin were increased with a 10 nM addition of P4. Dose-dependent decreases of [3H]Thymidine incorporation were witnessed in C2C12 myoblasts with the addition of P4. Myoblast treatment with MGA resulted in heightened myogenin mRNA levels. Data from this trial suggest that progestins may reduce satellite cell proliferation, having effects on carcass composition.

Application

Satellite cells provide a plethora of putative advances for the future. The degenerative disease muscular dystrophy results from exhaustion of satellite cells, resulting in loss of muscle and death. Current research proves that transplanted satellite cells form grafts into muscle and generate repair and self-renewal (Collins, 2006). Research remains in preliminary stages, but offers promising future applications.

Satellite cells also provide potential for production in the meat industry. Satellite cells incite muscle hypertrophy. Hypertrophy provides value to industry with larger muscle fiber diameters. Harnessing the potential of these muscle stem cells promises economic and medical advances for the future.
CHAPTER 4
RESULTS

23A2RafER\textsuperscript{DD2} Myoblasts Express Pax7 and the MRFs

Previous work from this lab identified potential use for 23A2RafER\textsuperscript{DD2} myoblasts as a model for satellite cell quiescence. These myoblasts over-express constitutive active Raf in an inducible manner, sending the cells into a reversible quiescent state (Reed et al., 2007). Pax7 is expressed in 23A2RafER\textsuperscript{DD2} myoblasts in concert with other proteins, such as the myogenic regulatory factors (MRFs). In order to assess these combinations, several costains of proliferating cells were performed using anti-Pax7, anti-Myf5, anti-MyoD, and anti-myogenin as the primary antibodies. Each plating trial was performed at least three times, enumerating positive cells in eight random microscopic fields at 200x. Figure 4-1A depicts myoblasts simultaneously immunostained for MyoD and Pax7. As indicated by the arrow, Pax7 immunopositive (+), MyoD immunonegative (-) cells exist as about 2% of the total cell population. 23A2RafER\textsuperscript{DD2} myoblasts also participate in asymmetric cell division, a feature of self-renewing cells. Figure 4-1B details the boxed portion of Figure 4-1A, revealing a pair of nuclei with Pax7 and MyoD colocalizing to one of the daughter cells.

Expression frequencies of Pax7 and MRF population are listed in Table 4-1 and reveal heterogeneity within the 23A2RafER\textsuperscript{DD2} myoblasts. Pax7 (+) cells comprise about 30% of the 23A2RafER\textsuperscript{DD2} population, indicating that nearly one-third of the myoblasts possess the satellite cell lineage marker. Interestingly, approximately 2% of the myoblasts contained Pax7 and were void for other members of the myogenic regulatory factor family, suggesting a minor self-renewing satellite cell population. A proposed model for the progression of a developing myoblast (Figure 4-2) describes a satellite cell as containing Pax7, and possibly Myf5. A portion
of the myoblast cell line reflects this protein expression pattern and corresponds to the Pax7 category in this diagram.

The myogenic regulatory factors indicate commitment down the myogenic pathway, expressions varying at different time points in the development of a myoblast. Myf5, perhaps the most naïve of this family, demonstrates a nuclear localization in 91% of the myoblast cells. Nuclear MyoD expression was evident in approximately 63% of cells, all of which were observed to contain Myf5. The “pre-myoblast” (Figure 4-2) describes a cell transitioning from the satellite cell compartment, still containing Pax7, but beginning to express proteins such as Myf5 and MyoD. Expression of these proteins characterize a cell progressing to the myoblast stage. Myoblasts do not contain Pax7 and are positive for other markers from the myogenic regulatory factor family. Very few myogenin positive myoblasts were observed, less than one percent of the population. Myogenin dictates terminal differentiation, and the formation of muscle from myoblasts. Absence of myogenin reveals a fertile myoblast population. Figure 4-3 depicts that 23A2RafER\textsuperscript{DD2} myoblasts fulfill each stage in the development of a myoblast.

\textbf{23A2RafER\textsuperscript{DD2} Protein Expression Provides a Mechanism for Adoption of Divergent Fates}

The presence of Pax7 in 23A2RafER\textsuperscript{DD2} myoblasts and the existence of cells containing Pax7 that are void of the myogenic regulatory factors duplicates all phases of satellite cell biology. Examination of the number of Pax7 (30%) and MyoD (63%) cells suggests that asymmetric division may give rise to the committed myoblast population. Support for this concept is found in Figure 4-4. Proliferating myoblasts were fixed and immunostained for Pax7 and Myf5. Myf5 localization to each cell in this doublet ensures the myoblast lineage. Pax7 expression in just one nucleus reveals divergent fates for each cell.
E2F5 and LEK1 Expression Plays a Role in Myoblast Quiescence

Work with primary cultures of satellite cells from this lab established similarities between this in vitro system and the isolated cells (Reed et al., 2007). 23A2RafER^{DD2} myoblasts express an inducible chimeric Raf protein. Induction of high levels of Raf allows these cells to exit the cell cycle into a quiescent state. Two proteins of interest were identified in this process: E2F5 and LEK1. Proliferating 23A2RafER^{DD2} cells express E2F5 in a cytoplasmic pattern, with LEK1 localizing to the nucleus and cytoplasm. With elevated levels of active Raf and pERK1/2, 23A2RafER^{DD2} myoblasts show a translocation of E2F5 and LEK1 to nuclear destinations, establishing a role for these proteins in myoblast quiescence. Isolated primary cultures of murine satellite cells from hindlimb muscles exhibit events that mirror those demonstrated by the 23A2RafER^{DD2} myoblasts. Satellite cells were fixed at culture time points that reflect a non-dividing state or actively progressing through the cell cycle. The satellite cells were immunostained for E2F5, LEK1, M-cadherin, and Pax7. In non-dividing satellite cells, E2F5 is retained in the nucleus (Figure 4-5). After three days in culture, cells are proliferating and E2F5 is located exclusively in the cytoplasm. A similar localization pattern is demonstrated for LEK1 (Figure 4-6).

E2F5 and LEK1 Are Expressed in vivo

To examine protein expression patterns in vivo, male murine tibialis anterior (TA) and bovine infraspinatus (INF) muscles were removed and frozen in OCT. Muscle cryosections were cut at 8µm and immunostained for LEK1, Pax7, myogenin, and E2F5. Adult mouse muscle contains a population of satellite cells that express LEK1 (Figure 4-7). However, Pax7 (+) cells void of LEK1 also exist. These results provide evidence for heterogeneity in the satellite cell population.
LEK1 may play a role in cell cycle arrest and fate decisions in vivo. Cryosections of six week old mouse TA muscle were immunostained with anti-LEK1 and anti-myogenin. A putative mitotic pair is depicted in Figure 4-8. LEK1 appears to have a nuclear and cytoplasmic localization characteristic of an actively proliferating satellite cell. LEK1 co-expression with myogenin suggests a role for LEK1 in the cell cycle exit necessary for myoblast terminal differentiation. The distribution pattern is not unique to mouse. Cryosections from calf *infraspinatus* muscle was immunostained for LEK1 and Pax7 (Figure 4-9). Pax7 localizes preferentially to one nucleus, with LEK1 predominately in the sister cell.

A similar but unique role for E2F5 in muscle is proposed. Mouse TA cryosections were stained for Pax7 and E2F5 (Figure 4-10). The satellite cell identified by Pax7 retains E2F5. The putative daughter cell in the perpendicular plane is deficient in both proteins. The asymmetrical localization of E2F5 was witnessed in all observations. In this representative photo, an E2F5 (+), Pax7 (-) cell demonstrates that E2F5 expression is not limited to the satellite cell compartment. E2F5, like LEK1, may demarcate non-dividing cells in general.

**E2F5 and LEK1 Localize to Myonuclei**

E2F5 and LEK1 localize to satellite cell nuclei, as well as other nuclei found in the muscle. In order to determine the nature of these nuclei, murine cryosections were immunostained for these proteins along with dystrophin, to distinguish the barriers between individual muscle fibers (Figure 4-11). Nuclei found within the individual myofiber boundaries are referred to as myonuclei. Nuclei adjacent to the outer dystrophin boundary are defined as putative satellite cells. As shown in Figure 4-11, E2F5 (A) and LEK1 (B) are expressed in nuclei on either side of the dystrophin barrier, in putative satellite cells as well as myonuclei. These results support a role for the proteins in cell cycle arrest.
E2F5 and LEK1 Expression Varies with Aging

To evaluate E2F5 and LEK1 expression patterns as a function of age, three young female mice from young (3-4 weeks) and adult (9 months) age groups were sacrificed for collection of TA muscles. Muscle samples from female mice did not provide reproducible results with the Pax7 antibody due to the high background staining. This ‘mouse on mouse’ effect is commonly witnessed when using mouse antibodies on mouse samples, which contain high levels of mouse antigens that elicit non-specific antibody binding. The use of a ‘mouse on mouse’, or M.O.M. kit to block these non-specific binding sites was attempted, however results showed no improvement. Due to time constraints, the Pax7 costain was discontinued.

Table 4-2 demonstrates the comparison of protein expression between young and adult murine TA myonuclei. In young samples, approximately 32% of myonuclei revealed E2F5 staining. This number increased (p < .01) to 63% when examining adult muscle. Similarly, LEK1 expression revealed a significant increase (p < .01) in the transition between young and old. Young mouse TA samples immunostained positive for LEK1 in about 60% of myonuclei, increasing to 78% in adult samples. Muscle growth was incomplete in the young mice. Increased levels of E2F5 and LEK1 in the adult suggest a role for these proteins in maintenance of cell cycle exit for the myonuclei of the fully-grown animals.

To assess the distribution of Pax7, E2F5, and LEK1 proteins in bovine muscle, cryosections of INF muscles from 4 young (less than one year) and 4 aged (11-12 years) samples were analyzed. Three separate observations determined that approximately 11% of nuclei present in calf muscle are satellite cells (Table 4-3). As the animal ages, satellite cell number declines because they are no longer needed for participation in rapid growth. Five percent of aged bovine muscle nuclei contained Pax7 (Table 4-3). The decrease in satellite cell number with age is in agreement with data collected for other species.
Aged bovine myonuclei were 14% E2F5 (+) and 21% LEK1 (+). Young bovine myonuclei contained 51% E2F5 and 31% LEK1. These values represent a significant difference over age for each protein (p < .01). As with myonuclei, satellite cell comparisons between young and old bovine muscle samples reveal a decrease in E2F5 and LEK1 percentages with age. E2F5 was expressed in 50% of young satellite cell nuclei and in 11% of old satellite cell nuclei. LEK1 was expressed in 38% of young satellite cell nuclei and in 17% of old satellite cell nuclei (Table 4-4). The low numbers of aged satellite cells expressing these markers of quiescence indicate a possible decrease in need for these proteins over time.

**Sarcoplasmic E2F5 Is Expressed in Young Type I Myofibers**

In the process of completing this study, an interesting result was observed. While staining nuclei in young bovine INF cryosections, E2F5 antibody also produced staining in the sarcoplasm of about half of the myofibers. The pattern of this expression resembled that of fiber type staining. Young bovine INF cryosections were simultaneously probed for E2F5 and myosin heavy chain of either type I or type IIa (Figure 4-12). Results show that all type I fibers express E2F5 in the sarcoplasm (A) and that type IIa fiber sarcoplasm is void of this protein (B). Myosin heavy chain type IIa expression is distinct from E2F5 in fibers. This eliminates the concern of a species cross-reactivity between these antibodies, as both myosin heavy chain antibodies were developed identically. Adult murine cryosections displayed only nuclear E2F5 staining, proving the sarcoplasmic expression pattern unique to young myofibers (Figure 4-13).

E2F5 antibody staining was specific to this particular protein. Similarities between the E2F4 and E2F5 proteins cause concern for non-specific detection. Preabsorption of E2F5 ensured this specificity. GST-E2F5 protein was attached to glutathione 4B sepharose beads. A working concentration of E2F5 antibody was passed over this column to absorb the anti-E2F5
antibody, and the product was used on young bovine INF cryosections. E2F5 staining retained its sarcoplasmic staining pattern in the control and produced no staining in the preabsorbed test (Figure 4-14).
23A2RafER^{DD2} myoblasts express Pax7 and MyoD. Proliferating 23A2RafER^{DD2} myoblasts were incubated with \(\alpha\)-Pax7 and \(\alpha\)-MyoD primary antibodies for immunofluorescent detection and photographed at 630x (A). Hoechst dye was used for nuclei visualization. Part B provides greater magnification of the boxed region.
Table 4-1. Profile of the 23A2RafER^DD2 murine myoblast cell line by percentages of Pax7 and myogenic markers

<table>
<thead>
<tr>
<th>Protein</th>
<th>% of Population</th>
<th>n</th>
<th>Total Cells Evaluated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pax7</td>
<td>30.0 ± 0.6</td>
<td>6</td>
<td>356</td>
</tr>
<tr>
<td>Myf5</td>
<td>91.1 ± 1.5</td>
<td>4</td>
<td>235</td>
</tr>
<tr>
<td>MyoD</td>
<td>62.6 ± 2.6</td>
<td>5</td>
<td>274</td>
</tr>
<tr>
<td>myogenin</td>
<td>0.5 ± 0.3</td>
<td>3</td>
<td>360</td>
</tr>
<tr>
<td>Pax7(+),Myf5/MyoD(-)</td>
<td>2.1 ± 1.6</td>
<td>3</td>
<td>524</td>
</tr>
</tbody>
</table>

Values represent mean ± SE. Eight random fields from each plating trial (n) of proliferating cells were counted. Total represents the total number of cells observed for presence of a particular protein.
Figure 4-2. Protein markers describe the developmental progression of a myoblast.
Figure 4-3. 23A2RafERDD2 myoblasts represent all stages in myoblast development. Proliferating 23A2RafERDD2 myoblasts were fixed and immunostained for different combinations of Pax7, Myf5, and MyoD. Hoechst dye was used for nuclei visualization at 630x. Arrows indicate representative nuclei for each compartment.
Figure 4-4. Myf5 expression in an asymmetrically dividing 23A2RafER<sup>DD2</sup> doublet provides a mechanism for divergent fates. Proliferating 23A2RafER<sup>DD2</sup> cells were immunostained for Pax7 and Myf5. Hoechst dye was used for nuclei visualization.
Figure 4-5. E2F5 expression in isolated murine satellite cells. Primary cultures were established from adult male murine hind limb muscle and immunostained for E2F5 and M-cadherin. Hoechst dye was used for visualization of nuclei. Representative photomicrographs at 630x shown (adapted from Reed et al., 2007).
Figure 4-6. LEK1 expression in isolated murine satellite cells. Primary cultures were established from adult male murine hind limb muscle and immunostained for LEK1 and Pax7. Hoechst dye was used for visualization of nuclei. Representative photomicrographs at 630x shown (adapted from Reed et al., 2007).
Figure 4-7. Murine cryosections exhibit Pax7 and LEK1. Immunocytochemical staining reveals nuclear Pax7 and LEK1 expression from an 8µm adult male murine TA muscle cryosection at 630x. Arrow depicts a cell co-localizing the two proteins. Hoechst dye was used for nuclei visualization.
Figure 4-8. Myogenin and LEK1 colocalize asymmetrically in adjacent nuclei. Adult male murine TA muscle cryosections were cut at 8µm and immunostained for LEK1 and myogenin. Hoechst dye was used for nuclei visualization.
Figure 4-9.  Pax7 and LEK1 expression in young bovine *infraspinatus* muscle cryosections.  Eight micrometer tissue sections (less than three months) were immunostained for Pax7 and LEK1.  Hoechst dye was used to visualize nuclei.  Representative photomicrographs at 630x are shown.
Figure 4-10. E2F5 and Pax7 expression in adult murine TA muscle cryosections. Eight micrometer tissue sections were immunostained for Pax7 and E2F5. Hoechst dye was used to visualize nuclei. Representative photomicrographs at 630x are shown.
Figure 4-11. Adult murine TA cryosections reveal E2F5 and LEK1 in myonuclei and satellite position cells. Eight micrometer tissue sections were immunostained for E2F5 (A) and LEK1 (B) with dystrophin. Hoechst dye was used to visualize nuclei. Representative photomicrographs at 630x are shown.
Table 4-2. Comparison of young versus adult murine TA myonuclei protein profiles

<table>
<thead>
<tr>
<th>Protein</th>
<th>Young</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Adult</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of (+)</td>
<td>n</td>
<td>Total</td>
<td>Myonuclei</td>
<td>% of (+)</td>
<td>n</td>
<td>Total</td>
<td>Myonuclei</td>
</tr>
<tr>
<td>E2F5</td>
<td>31.7 ± 2.8&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3</td>
<td>1969</td>
<td>63.0 ± 2.5&lt;sup&gt;B&lt;/sup&gt;</td>
<td>3</td>
<td>996</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LEK1</td>
<td>59.5 ± 2.5&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3</td>
<td>2100</td>
<td>78.1 ± 2.7&lt;sup&gt;B&lt;/sup&gt;</td>
<td>3</td>
<td>1193</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values represent mean ± SE. Six random fields from each observation of n number of animals were counted. Total represents the total number of myonuclei enumerated. Different letters in each row represent a significant difference (p<.01).
Table 4-3. Percentage of satellite cells present in young versus aged bovine muscle cross sections

<table>
<thead>
<tr>
<th>Age</th>
<th>% Pax7(+)</th>
<th>n</th>
<th>Total Myonuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young</td>
<td>10.5 ± 1.1</td>
<td>3</td>
<td>558</td>
</tr>
<tr>
<td>Aged</td>
<td>5.3 ± 0.7</td>
<td>8</td>
<td>235</td>
</tr>
</tbody>
</table>

Values represent mean ± SE. Six random fields from each of n observations were counted. Total represents the total number of myonuclei enumerated.
Table 4-4. Percentage of E2F5 and LEK1 proteins in aged bovine *infraspinatus* satellite cells

<table>
<thead>
<tr>
<th>Protein</th>
<th>% of Protein (+) Satellite Cells</th>
<th>% of Protein (-) Satellite Cells</th>
<th>n</th>
<th>Total Pax7(+) Satellite Cells</th>
<th>Total Myonuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2F5</td>
<td>11.3 ± 3.2</td>
<td>88.7 ± 3.2</td>
<td>4</td>
<td>102</td>
<td>1410</td>
</tr>
<tr>
<td>LEK1</td>
<td>17.4 ± 4.3</td>
<td>82.6 ± 4.3</td>
<td>4</td>
<td>75</td>
<td>1448</td>
</tr>
</tbody>
</table>

Values represent mean ± SE. Six random fields from each observation of n number of animals were counted. Total represents the total number of myonuclei enumerated.
Figure 4-12.  Sarcoplasmic E2F5 in type I bovine (less than three months) *infraspinatus* muscle fibers. Eight micrometer tissue sections were immunostained for Myosin Heavy Chain type I (A) and IIa (B) with E2F5. Hoechst dye was used to visualize nuclei. Representative photomicrographs at 630x are shown.
Figure 4-13. Sarcoplasmic E2F5 is not expressed in aged bovine (11-12 years) *infraspinatus* muscle fibers. Eight micrometer tissue sections were immunostained for Myosin Heavy Chain type I (A) and IIa (B) with E2F5. Hoechst dye was used to visualize nuclei. Representative photomicrographs at 630x are shown.
Figure 4-14. Preabsorption of E2F5 ensures E2F5 antibody staining specificity. GST-E2F5 protein was attached to glutathione 4B sepharose beads. A working concentration of α-E2F5 antibody was incubated with the beads and used as primary antibody. Eight micrometer bovine (less than three months) infraspinatus cryosections were immunostained with the preabsorbed E2F5 antibody and anti-E2F5 as a control. Representative photomicrographs at 630x are shown.

α-E2F5
Preabsorbed E2F5
control
CHAPTER 5
DISCUSSION

Embryonic Myoblasts as a Model for Satellite Cell Quiescence

*In vitro* systems offer great potential for advances in research due to their stability and ease of use. Embryonic myoblasts show similarities to satellite cells. The cell line utilized in these experiments, 23A2RafER\textsuperscript{DD2}, was derived from embryonic mouse myoblasts. These cells contain a modified estrogen receptor fused to the Raf molecule. Dose-dependent treatment with 4-hydroxytamoxifen (4HT), an estrogen analog, affects proliferation in the myoblasts. High levels of induced Raf send these 23A2RafER\textsuperscript{DD2} myoblasts into a reversible quiescent state, similar to the quiescent state in which non-active satellite cells reside (Reed et al., 2007). Satellite cell isolations yield cells that are in the process of activation (Zammit, 2004). The possible induction and maintenance of a true quiescent state would offer great benefits over isolation of satellite cells in primary culture. 23A2RafER\textsuperscript{DD2} myoblasts can also be manipulated, proving benefits over observing *in vivo* samples.

However, in order to be useful, such cell lines must be validated against *in vivo* systems. To assess the characteristics of the 23A2RafER\textsuperscript{DD2} myoblasts population, immunostaining for Pax7, Myf5, MyoD, and myogenin was performed. Interestingly, this myoblast cell line demonstrated nuclear expression of Pax7 in 30% of cells. This anti-apoptotic protein, unique to satellite cells offers the potential for self-renewal and lends credibility to use of this cell line to mimic satellite cell behavior. MyoD and Myf5 expression identified undifferentiated, proliferating myoblasts. Combinations of these myogenic regulatory factors dictate stages of commitment during myogenesis. Myf5 and MyoD co-expression with Pax7 insinuates an activated, or proliferating satellite cell.
Coordination of these factors agrees with a model proposed by Relaix et al. (2006). This group proposes initial expression of Myf5 upon adoption of a myogenic fate, followed by MyoD and ultimately MRF4 and myogenin. Likewise, upon activation, Pax7 (+) satellite cells first express Myf5 followed by MyoD. Return of a satellite cell to a quiescent state requires the down-regulation of MyoD and Myf5. The 23A2RafER<sup>DD2</sup> myoblasts represent all categories of this model. Myf5 expression in 91% of cells insinuates that this marker acts as a gateway to other proteins. MyoD (+) cells all co-express Myf5.

A small portion of the 23A2RafER<sup>DD2</sup> myoblast population (approximately two percent) contains Pax7 protein and is void of myogenic regulatory factors. This possibly more naïve subset of the population offers the potential for self-renewal. Myogenin, a marker of terminal differentiation, was demonstrated in less than one percent of the population. About seven percent of the 23A2RafER<sup>DD2</sup> myoblasts were not defined by these markers. These cells could possibly contain Pax3, a paralog of Pax7 expressed in a subset of muscles, and also during embryogenesis. It is likely that this marker of more naïve satellite cells is expressed in embryonic myoblasts, due to their origin. Failures with several Pax3 antibodies hindered further classification of the 23A2RafER<sup>DD2</sup> myoblasts. While the 23A2RafER<sup>DD2</sup> population does not exclusively represent satellite cells due to their protein profile, a portion of this population remains viable for further comparisons.

Satellite cells may preserve their small population by participating in asymmetric cell division, or allocation of particular proteins, namely Numb, to only one cell of a dividing doublet. The cell that receives Numb, and most likely Pax7, maintains the satellite cell identity, replenishing the satellite cell compartment within muscle. The daughter cell that does not inherit Pax7 potentially becomes a myoblast and participates in growth or regeneration (Conboy and
Zammit et al. (2004) support the stochastic model and detail that satellite cells initially proliferate, expressing Pax7 and myogenic regulatory factors. After 72 hours in culture, this group found that a significant portion of satellite cells exist that are Pax7 positive and MyoD negative, insinuating that a portion of the population stops expressing myogenic regulatory factors to return to quiescence. It is likely that satellite cell fate determination functions as a combination of different forms of division.

23A2RafER$^{DD2}$ myoblasts demonstrate these patterns of division. By use of immunostaining, a mechanism for divergent fate adoption could be identified within this myoblast population. With asymmetric localization of Pax7 in a 23A2RafER$^{DD2}$ myoblast doublet, each member of the pair inherits Myf5. This myogenic regulatory factor dedicates the Pax7 (-) sister cell to become a myoblast. Myf5 expression in the Pax7 (+) cell demonstrates that this cell is an activated satellite cell. 23A2RafER$^{DD2}$ myoblasts may initially divide in a symmetric manner, with loss of Pax7 expression later occurring in one of the cells. The potential for adoption of divergent fates further cements the possibility for use of this cell line in analysis of satellite cell behavior.

Previous work by this lab (Reed et al., 2007) identified two proteins unique to the nuclei of quiescent 23A2RafER$^{DD2}$ myoblasts: E2F5 and LEK1. Nuclear localization of these proteins also was evident in quiescent isolated murine satellite cells. Non-quiescent cells expressed E2F5 in a nuclear fashion, while LEK1 expression was exhibited throughout the cytoplasm and nucleus. E2F5 or LEK1 expression was not exclusive to Pax7 (+) 23A2RafER$^{DD2}$ cells, indicating that myoblasts are capable of expressing these proteins, and that they are not unique to satellite cells.
This trend extends into mature muscle. In murine and bovine muscle cryosections, E2F5 and LEK1 expression was detected in both the nuclei of satellite cells and myonuclei. Satellite cell and myonuclei classification was determined by immuostaining with Pax7 and relation to the dystrophin barrier. Young bovine and murine myofibers demonstrated E2F5 expression in the sarcoplasm of type I fibers. It is possible that cytoplasmic expression of these proteins correlates with an embryonic or more naïve phenotype.

Similarities exist between the 23A2RafERDD myoblasts and an in vivo system, defined by behavior and protein expression. While this cell line does not mirror satellite cell activity exactly, it provides promising clues for further investigation of satellite cell biology. To further examine this relationship, expression of Raf in vivo could be analyzed. High levels of Raf in vivo may correlate with quiescence and the nuclear translocation of E2F5 and LEK1.

Identification of other differentially expressed proteins could add credibility to this model.

**E2F5 and LEK1 Function in Maintenance of Cell Cycle Exit**

*In vitro* experiments utilizing 23A2RafERDD cells demonstrated that nuclear localizations of E2F5 and LEK1 are unique to quiescence, possibly defining a role for these proteins in attainment or maintenance of this cell cycle exit. Nuclear translocation of these two proteins within 15 minutes of induced quiescence (Reed et al., 2007) provides evidence that E2F5 and LEK1 proteins may aid in the adoption of the quiescent state. Goodwin et al. (1998) reported that the early embryo exhibits the highest levels of LEK1 mRNA expression and that these levels decrease significantly after birth. 23A2RafERDD cells, of an embryonic origin, likewise exhibit high levels of LEK1 protein expression.

To substantiate this trend, murine and bovine mature muscle cryosections were probed for Pax7, E2F5, LEK1, and dystrophin, then analyzed. *In vivo* data demonstrate that LEK1 and E2F5 are present in adult muscle, in myonuclei as well as satellite cell nuclei. The divergent
localizations of these proteins across cell types insinuates multiple functions for these proteins. However, these functions center around cell cycle control. E2F5 and LEK1 expression in satellite cell nuclei supports the trend of protein expression established by the myoblast cell line. Satellite cells in mature muscle exist in a quiescent state. Therefore, E2F5 and LEK1 may not only be involved with entrance into this quiescent state, but also with maintenance.

E2F5 and LEK1 presence in myonuclei suggests a more universal role for these proteins in cell cycle exit. Terminally differentiated cells have exited the cell cycle, yet are not quiescent. These cells do not have the potential to reenter the cell cycle. Therefore, E2F5 and LEK1 participate in attainment or maintenance of cell cycle exit, but not particularly quiescence. In accordance, Gaubatz et al. (2000) found that E2F5 functions as a transcriptional repressor. E2F5 and LEK1 possibly interact to direct or maintain cell cycle exit (Reed et al., 2007). However, more LEK1 (+) myonuclei and satellite cell nuclei are detected than E2F5 (+) nuclei (p < .01), suggesting different roles for each of these proteins. LEK1 possibly has a longer half-life than E2F5 or has a more persistent role in the maintenance of cell cycle exit.

**E2F5 and LEK1 Affect Cell Fate Decisions**

A method of putative fate determination utilized by satellite cells involves asymmetric partitioning of proteins or genetic material to influence cell fate. For example, Numb generally localizes to only one nucleus of a dividing doublet. Potentially, the cell that adopts Numb and possibly Pax7 remains as a satellite cell and has the capability of returning to quiescence. The cell void of Pax7 will develop as a myoblast, ultimately reaching terminal differentiation and exiting the cell cycle (Conboy and Rando, 2002). Shinin et al. (2006) found evidence of template strand cosegregation in satellite cell derived cultures through BrdU labeling, which creates an immortal strand to protect the genome from DNA replication errors. The immortal
DNA strands were found together with Pax7 and Numb, and these cells did not differentiate. In this manner, cell fates may be assigned as cells exit the cell cycle.

Upon observation of dividing satellite cell doublets in murine and bovine cryosections, E2F5 and LEK1 exhibited differing trends of expression. E2F5 protein localized to the same nucleus that inherited Pax7 (Figure 5-1). Nuclear localization of E2F5 most likely predicts exit into quiescence for this satellite cell. Expanding on previous data, perhaps E2F5 presence in myonuclei insinuates that these nuclei were once satellite cells and lost their Pax7 identity over time or through muscle growth and repair. E2F5 could promote maintenance of cell cycle exit or persist due to a long half-life.

LEK1 conversely localized to the cell destined to become a myoblast in a dividing doublet, colocalizing with myogenin (Figure 5-1). Myogenin indicates a myoblast awaiting terminal differentiation. Nuclear localization of LEK1 therefore reflects exit from cell cycle, but with terminal differentiation. However, satellite cells express LEK1 in vivo. LEK1 expression could possibly indicate a universal withdrawl from cell cycle. Perhaps LEK1 allocates to a satellite cell that is not immediately returning to quiescence. Though this data crosses species, taken together, it demonstrates a similar motif: a role for LEK1 in cell cycle arrest, particularly in relation to determination of terminal differentiation in myoblasts and maintenance of quiescence in satellite cells.

To examine these theories further, immunohistochemistry could be performed to simultaneously immunostain for Pax7, E2F5, and LEK1. However, the antibodies for E2F5 and LEK1 found to be reliable by this lab are both rabbit polyclonal. At this stage, such a multidetection assay is not possible due to cross-reactivity. Nevertheless, E2F5 and LEK1 play unique roles in the maintenance of cell cycle exit within muscle.
E2F5 and LEK1 Expression in Satellite Cell Nuclei Varies with Age

The literature has determined that satellite cells decrease in number over the lifespan of a mouse. In the first few weeks after birth, the numbers of satellite cells in rodent hindlimb muscle declines from about 30% of total nuclei to less than 5% (Schultz and McCormick, 1994). Studies of this nature have not been reported in livestock. Samples from a four-week-old calf determined that approximately 11% of nuclei present in calf muscle are satellite cells. As the animal ages, satellite cell number declines because they are no longer needed for participation in rapid growth. Five percent of aged bovine muscle nuclei contained Pax7. The decrease in satellite cell number with age is in agreement with data collected for other species.

Original experimental design detailed examination of young (3-4 week old) murine, adult (9 month old) murine, young (less than three months) bovine, and aged (11-12 years) bovine samples to compare differences across age and species. Bovine samples were obtained from other researchers in this department, therefore the conditions for procurement of these samples could not be controlled. Young bovine samples were collected and aged for 14 days, most likely causing a decrease in protein content. Therefore, these results are mentioned, but not used in concrete comparisons. However, young bovine samples displayed significantly more (p < .01) E2F5 (+) and LEK1 (+) nuclei than aged bovine samples, indicating that protein was not completely lost. Repetition of this trial with immediate preservation of samples would verify this data. Also, consistency with ages could provide a more complete model and direct comparison between species.

Previous work utilized male mice, providing predictable results for Pax7 immunostaining. However, the male mice inflicted injury and stress upon one another, leading to the decision to switch to female specimens. Female mouse samples used in conjunction with mouse Pax7 antibody produced high levels of background staining, yielding inconsistent results. Due to time
constraints, Pax7 and satellite cell data was not collected for murine samples. Pax7, E2F5, and LEK immunostaining should be repeated in male mice to verify the trends observed in bovine samples.

Young bovine samples contained 50% E2F5 (+) and 38% LEK1 (+) satellite cells. These numbers decreased to 11% and 17%, respectively (p < .01 per protein) in aged samples. E2F5 and LEK1 seem to play roles in cell cycle exit and quiescence, so it is expected to see an increase in these numbers, because more satellite cells are quiescent in older age. It is possible that with age, satellite cells lose their requirement for these proteins. Depth of quiescence may contribute to the fact the older satellite cells take longer to activate and participate in muscle repair (Schultz and Lipton, 1982). Lack of E2F5 or LEK1 could contribute to this state. Further investigation into this area could broaden understanding of satellite cell activity in relation to aging. Perhaps injury in an aged animal would produce an up-regulation of E2F5 and LEK1 as satellite cells activate, proliferate, and return to quiescence.

**E2F5 and LEK1 Expression in Myonuclei Varies with Age**

Though crossing species, comparisons between protein expression levels of the young murine, young bovine, adult murine, and aged bovine myonuclei could reveal information about the aging process in terms of fluctuations in levels of E2F5 and LEK1. Less myonuclear E2F5 and LEK1 expression was detected in young mouse muscle when compared with adult mouse muscle (p < .01). These results insinuate that both proteins are up-regulated as the animal ceases to grow, suggesting a role for E2F5 and LEK1 in cell cycle exit and terminal differentiation.

Adult mice are not equivalent with aged cows and demonstrate different timepoints in the lifecycle. Adult mouse immunohistochemistry revealed 63% E2F5 (+) and 78% LEK1 (+) myonuclei. These numbers decrease dramatically in aged bovine samples. Fourteen percent of aged bovine myonuclei were E2F5 (+) and 21% revealed LEK1. Likewise, old bovine samples
contain fewer numbers of E2F5 (+) and LEK1 (+) nuclei, suggesting a decrease in expression of these proteins as a function of age. These results further support the theory of an age-dependent regulation of E2F5 and LEK1 expression.

Differences suggest fluid levels for E2F5 and LEK1 expression in developing muscle, perhaps reacting to differing needs of myonuclei to maintain cell cycle exit over time. A proposed explanation is detailed as follows. Young myonuclei express low levels of E2F5 and LEK1 as they have just exited the cell cycle and the muscle develops. As the animal grows, more myonuclei have been added to the mature muscle. Terminal differentiation and cell cycle exit explains the greater number of E2F5 (+) and LEK1 (+) myonuclei in adult mouse muscle (p < .01). As the animal ages, E2F5 and LEK1 protein in myonuclei decreases dramatically. It is possible that after extended periods of cell cycle exit, the two proteins are no longer required by the muscle to assist in maintenance of cell cycle exit. Another possibility exists that E2F5 and LEK1 regulation occurs differently across species, explaining the various levels of E2F5 and LEK1 protein expression.

**Type I Myofibers in Young Animals May Exhibit an Embryonic Phenotype**

E2F5 and LEK1 expression in this study were detected nuclearly in all samples. However, an interesting phenomenon was observed in young bovine tissue samples. E2F5 protein was expressed throughout the sarcoplasm of about half of the myofibers, as well as in nuclei. This immunostaining pattern resembled fiber type staining, so myosin heavy chain type I and Ila antibodies were used in E2F5 costains. E2F5 sarcoplasmic protein expression was detected in type I myofibers only. Protein expression levels can vary in a fiber type-specific manner (reviewed by Pette and Staron, 2001).

In a young animal, type I fibers are still growing and developing. Proliferating satellite cells in culture and proliferating or growth-arrested 23A2RafERDD2 cells express E2F5
cytoplasmically (Reed et al., 2007). The developing type I fibers of tissue sections likewise expressed E2F5 in a cytoplasmic pattern. This unique staining pattern in young myofibers may result from the persistence of an embryonic phenotype in the type I fibers. Quiescent satellite and 23A2RafERDD2 cells demonstrate nuclear localizations for E2F5 (Reed et al., 2007). With age, E2F5 expression is detected solely in the satellite cell nuclei and myonuclei of aged bovine samples, suggesting nuclear translocation of the sarcoplasmic protein with age and cell cycle exit. The nuclear translocation of E2F5 upon entrance of cell cycle exit exhibited by 23A2RafERDD2 myoblasts could model the events that occur within living muscle.
Figure 5-1. Proposed model for E2F5 and LEK1 localization in asymmetric satellite cell division
CHAPTER 6
IMPLICATIONS

Understanding of satellite cell biology and skeletal muscle development has applications that span the gamut of medical therapies and advances. However, a less developed area of research involves application of this information towards the livestock industry. Satellite cells are responsible for all postnatal muscle growth. Residing in a quiescent, or resting state in mature muscle, these cells require environmental cues for activation. Therefore muscle growth, or hypertrophy in livestock occurs due to the activation and proliferation of satellite cells. Harnessing the potential of these satellite cells promises economic benefits for producers with greater meat production.

A particular area of interest involves improvement of meat yield and quality from aged animals. Satellite cells lose regenerative potential in older animals, most likely due to an inability to exit the quiescent state. Understanding of the mechanisms and proteins that contribute to depth of quiescence would yield new directions for such research. Environmental signals initiate exit from the quiescent state. It seems that failure of environmental cues plays the greatest role in delays for activation of satellite cells in older animals. Current research examines the affects of various supplements on meat production. Future work could focus on accretion of muscle through mediation of satellite cell effectors.

The study of satellite cells and muscle development offers great potential for the meat industry. Research must approach the goal of product improvement from two directions: proteomic and applied. Deep understanding of muscle development at a molecular biology level and application of this information in trials with livestock promise benefits for the growing world population as well as producers.
LIST OF REFERENCES


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BIOGRAPHICAL SKETCH

Sara Elizabeth Ouellette was born December 11, 1982, to two University of Florida Gators, Mary and Leo Ouellette. At seven years of age, Sara was blessed with a younger sister, Megan. Sara attended school in the Fort Lauderdale area of Florida for elementary, middle, and high school, focusing on science and math through Advanced Placement courses. In Fall 2001, Sara fulfilled the family legacy and became a part of the Gator Nation at the University of Florida. Following the pre-veterinary medicine track, Sara matriculated cum laude with her Bachelor of Science in animal sciences with animal biology focus in May 2005. For the next two years, Sara worked as a graduate assistant to achieve her Master of Science in animal sciences under Dr. Sally Johnson. During this time, Sara also had the fortunate experience of supervising a staff of undergraduate Resident Assistants as a Graduate Hall Director in Graham Residence Hall on campus. In the Fall of 2007, Sara will be attending a college of veterinary medicine in pursuit of a Doctorate of Veterinary Medicine in 2011.