Regulation of Satellite Cells During Skeletal Muscle repair and Regeneration

by

Rajani M. George

A Dissertation Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy

Approved January 2012 by the Graduate Supervisory Committee:

N. Jeanne Wilson-Rawls, Chair
J. Alan Rawls
Kenro Kusumi
G. Kerr Whitfield

ARIZONA STATE UNIVERSITY

May 2012
ABSTRACT

Postnatal skeletal muscle repair is dependent on the tight regulation of an adult stem cell population known as satellite cells. In response to injury, these quiescent cells are activated, proliferate and express skeletal muscle-specific genes. The majority of satellite cells will fuse to damaged fibers or form new muscle fibers, while a subset will return to a quiescent state, where they are available for future rounds of repair. Robust muscle repair is dependent on the signals that regulate the mutually exclusive decisions of differentiation and self-renewal. A likely candidate for regulating this process is NUMB, an inhibitor of Notch signaling pathway that has been shown to asymmetrically localize in daughter cells undergoing cell fate decisions. In order to study the role of this protein in muscle repair, an inducible knockout of Numb was made in mice. Numb deficient muscle had a defective repair response to acute induced damage as characterized by smaller myofibers, increased collagen deposition and infiltration of fibrotic cells. Satellite cells isolated from Numb-deficient mice show decreased proliferation rates. Subsequent analyses of gene expression demonstrated that these cells had an aberrantly up-regulated Myostatin (Mstn), an inhibitor of myoblast proliferation. Further, this defect could be rescued with Mstn specific siRNAs. These data indicate that NUMB is necessary for postnatal muscle repair and early proliferative expansion of satellite cells. We used an evolutionary compatible to examine processes controlling satellite cell fate
decisions, primary satellite cell lines were generated from *Anolis carolinensis*. This green anole lizard is evolutionarily the closest animal to mammals that forms de novo muscle tissue while undergoing tail regeneration. The mechanism of regeneration in anoles and the sources of stem cells for skeletal muscle, cartilage and nerves are poorly understood. Thus, satellite cells were isolated from *A. carolinensis* and analyzed for their plasticity. Anole satellite cells show increased plasticity as compared to mouse as determined by expression of key markers specific for bone and cartilage without administration of exogenous morphogens. These novel data suggest that satellite cells might contribute to more than muscle in tail regeneration of *A. carolinensis*. 
DEDICATION

This work is dedicated to my parents who have been my unfailing supporters while I worked my way through graduate school. This work would have been impossible without them. Thank you for being there for me in more ways than I can count.
ACKNOWLEDGMENTS

I would like to thank my committee members for all their guidance and support. I would especially like to thank Dr. Wilson-Rawls for mentoring me and and working tirelessly to make sure that I try to better myself every day. I would like to thank Dr. Rawls for all his scientific input and advice for this project, Dr. Kusumi for all his guidance and for making sure that I had a comprehensive understanding of the material and Dr. Whitfield for all his positive comments and input throughout the project.

I would like to thank all the graduate students that supported me in my graduate career. Dr. Brian Beres for his support when I first joined the lab and continued friendship through the years. Dr. Douglas Anderson for all his encouragement, scientific input and suggestions and Megan Rowton for her support through the stresses of graduate school. I would also like to thank all the students that helped with this work, Lauren Geiger, Erik Rogers, Jeramy Bullis, Erik Lougher, Walter Eckalbar, Glenn Markov, Amanda Mulia and Elizabeth Hutchins.

Finally, I would like to thank all the family and friends that provided encouragement and support these last few years.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>LIST OF TABLES</th>
<th>vi</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>vii</td>
</tr>
<tr>
<td>CHAPERS</td>
<td></td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Signaling pathways in development</td>
<td>2</td>
</tr>
<tr>
<td>Embryonic muscle development</td>
<td>6</td>
</tr>
<tr>
<td>Adult skeletal muscle and satellite cell mediated repair</td>
<td>9</td>
</tr>
<tr>
<td>Muscle regeneration</td>
<td>14</td>
</tr>
<tr>
<td>ROLE OF NUMB IN SATELLITE CELL MEDIATED MUSCLE REPAIR</td>
<td>18</td>
</tr>
<tr>
<td>Introduction</td>
<td>18</td>
</tr>
<tr>
<td>Results</td>
<td>21</td>
</tr>
<tr>
<td>Discussion</td>
<td>36</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>39</td>
</tr>
<tr>
<td>CHARACTERIZING SATELLITE CELLS IN THE REPTILE ANOLIS CAROLINENSIS</td>
<td>43</td>
</tr>
<tr>
<td>Introduction</td>
<td>43</td>
</tr>
<tr>
<td>Results</td>
<td>46</td>
</tr>
<tr>
<td>Discussion</td>
<td>60</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>62</td>
</tr>
<tr>
<td>CONCLUSIONS</td>
<td>65</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>69</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: Candidate gene QRT-PCR on <em>Numb</em> deficient satellite cells</td>
<td>32</td>
</tr>
<tr>
<td>2: Candidate gene QPCR for satellite cell markers</td>
<td>50</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: Satellite cell binary cell fate decision</td>
<td>3</td>
</tr>
<tr>
<td>2: Genetic regulation of satellite cell differentiation</td>
<td>11</td>
</tr>
<tr>
<td>3: Impaired regeneration in Numb deficient muscle</td>
<td>22</td>
</tr>
<tr>
<td>4: Isolation and genotyping of satellite cell lines</td>
<td>24</td>
</tr>
<tr>
<td>5: Differentiation of Numb deficient satellite cells</td>
<td>27</td>
</tr>
<tr>
<td>6: Numb deficient satellite cells do not proliferate</td>
<td>30</td>
</tr>
<tr>
<td>7: Numb deficient satellite cells do not undergo increased apoptosis</td>
<td>31</td>
</tr>
<tr>
<td>8: RNAi knockdown of Mstn rescues proliferation in Numb deficient satellite cells</td>
<td>35</td>
</tr>
<tr>
<td>9: Satellite cells from Anolis carolinensis express Pax7</td>
<td>47</td>
</tr>
<tr>
<td>10: Proliferation rates of anole and mouse satellite cells</td>
<td>51</td>
</tr>
<tr>
<td>11: Differentiation of mouse and anole satellite cells</td>
<td>53</td>
</tr>
<tr>
<td>12: Anole satellite cells express chondrogenesis and osteogenesis marker genes</td>
<td>57</td>
</tr>
<tr>
<td>13: Anole satellite cells form calcified nodules in osteogenic differentiation medium</td>
<td>59</td>
</tr>
</tbody>
</table>
CHAPTER 1
INTRODUCTION

Skeletal muscle is a terminally differentiated tissue that is capable of repairing itself after injury. A quiescent muscle-specific population of stem cells, called satellite cells are the primary cell type that contributes to skeletal muscle repair (Mauro, 1961, Muir et al., 1965, Schultz et al., 1978, Sherwood et al., 2004). When skeletal muscle is damaged, satellite cells become activated and proliferate (Cooper et al., 1999). Most cells within this expanded pool of satellite cells will continue to differentiate and contribute to the damaged myofiber (Collins et al., 2005, Gurley and Alvarado, 2008). However, a few cells will return to a quiescent state to repopulate the healed myofiber with satellite cells to repair future damage (Beauchamp et al., 2000). Thus, activated satellite cells have to make an important binary cell fate decision to differentiate or return to quiescence (Figure 1). The molecular mechanisms controlling this process of activation, proliferation, differentiation and returning to quiescence are not completely understood. The characterization of signaling pathways that determine satellite cell function represents a critical step towards expanding our knowledge of the molecular processes controlling the musculoskeletal system. Further understanding of the cellular and molecular processes by which muscle is able to repair or regenerate itself will improve our ability to develop clinical solutions for myopathies.
Signaling pathways in development

A conserved set of signal transduction pathways play an important role during development and progenitor cell fate decisions. One such important pathway is Notch signaling. In mammals, the Notch family consists of four proteins, NOTCH1, NOTCH2, NOTCH3 and NOTCH4 that have both an overlapping and unique expression pattern in tissues (Chiba, 2006, Fiuza and Arias, 2007). Upon binding with its ligands, Deltalike 1 (DLL1), DLL3 and DLL4 or Jagged1 and Jagged2, the extracellular domain of Notch protein is cleaved by a metalloprotease, TACE (Bettenhausen et al., 1995, Lindsell et al., 1995, Shawber et al., 1996, Dunwoodie et al., 1997, Shutter et al., 2000, Brou et al., 2000). This truncated protein is subsequently cleaved by γ-secretase (Fortini, 2002), and results in the formation of the Notch Intracellular Domain (NotchICD), which translocates into the nucleus. The CSL family of transcription factors (CBF1/RBPJκ, Suppresor of Hairless, Lag1) is associated with co-repressors (e.g. N-CoR, SHARP, CtBP) (De Strooper et al., 1999, Lai, 2002, Kovall and Hendrickson, 2004, Kovall, 2007). Upon interaction with NotchICD, these co-repressors are replaced by co-activators like Mastermind/MAML and p300/CBP leading to transcription of target genes
When adult skeletal muscle is injured, satellite cells resident on the myofiber that are normally quiescent become activated and undergo proliferative expansion. At this point, they have to make a binary cell fate decision to either progress along the myogenic differentiation pathway to become myocytes to fuse with the damaged myofiber or return to quiescence to re-populate the healed myofiber in preparation for future rounds of damage.

Figure 1: Satellite cell binary cell fate decision
(McElhinny et al., 2008). Notch target genes include *Hes* (Hairy enhancer of split) and *Hey* (Hairy Related Transcription factor) family of transcription factors (Nakagawa et al., 2000, Iso et al., 2003, Kageyama et al., 2007). This cascade of signal transduction events regulate cell fate decisions (Lai, 2004, Kopan and Ilagan, 2009).

NUMB is an important modulator of Notch signaling that have been shown to be involved in progenitor cell functions in non-muscle lineages (Uemura et al., 1989, Zhong et al., 2000). NUMB is cytoplasmically localized and has four protein isoforms that are generated by the alternative splicing of two exons. The first exon is within the phosphotyrosine binding domain (PTB) and the second exon is found in the proline rich region (PRR) (Dho et al., 1999). The protein products of these alternatively spliced transcripts, designated as NUMB65, NUMB66, NUMB71 and NUMB72 have been shown to have varying biological functions. For example, work in neuronal cell types has shown that NUMB71 and NUMB72 promote proliferation whereas NUMB65 and NUMB66 promote differentiation (Verdi et al., 1999, Pedersen et al., 2002, Toriya et al., 2006, Bani-Yaghoub et al., 2007). The PTB domain of NUMB has been shown to be responsible for potentiating the recruitment of the E3 ubiquitin ligase, Itch, to the NOTCH intracellular domain thus targeting NOTCH for degradation (McGill and McGlade, 2003, McGill et al., 2009). Recent work has also shown specificity of NUMB-Notch interactions. All
four NUMB isoforms target NOTCH1, some can inhibit NOTCH2, but none of the Numb proteins can inhibit Notch3 (Beres et al., 2011).

In addition to inhibiting NOTCH, NUMB has also been shown to interact with proteins involved in other signaling pathways and cellular processes. NUMB has been shown to ubiquitinate GLI1, the transcription factor downstream of Sonic Hedgehog signaling, an important signaling pathway that specifies a myogenic fate during early development (Bumcrot and McMahon, 1995, Marcotullio et al., 2006). NUMB has also been shown to be involved in cell migration by interacting with TrkB, the receptor for the chemotactic protein BDNF and targets the receptor protein for endocytosis (Mousavi and Jasmin, 2006, Zhou et al., 2011).

Another signaling pathway that plays an important role in cell fate regulation is the TGF-β (Transforming Growth Factor) superfamily of structurally related, secreted, dimeric proteins. This signaling pathway acts through serine/theronine kinases receptors. Binding of TGF-β family members to the type II receptor leads to its phosphorylation followed by recruitment and phosphorylation of a type I co-receptor thus creating an activated receptor complex. This complex then directly phosphorylates transcription factors known as (p)SMADs. Dimerized pSMADs form a complex with SMAD4 and translocate into the nucleus to initiate target gene expression. The TGF-β family include important signaling molecules such as bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), Activins and TGF-βs. Myostatin (MSTN) (GDF-8) is an
important member of this family. Dimeric MSTN binds to activin type IIB receptor (ACTRIIB) and recruits the type I activin-like kinase (ALK)4 or ALK5. This activated receptor complex then phosphorylates the transcription factors SMAD2 and SMAD3, leading to target gene expression (Rebbapragada et al., 2003). Another important member of the TGF-β family of signaling molecules, BMP2, which binds to its receptors such as BMPRIa, BMPRIIa causing phosphorylation of SMAD1 and SMAD4 (Nohe et al., 2002).

These important signaling pathways are involved in signaling events leading to specification, commitment and lineage specific differentiation of progenitor populations of cells. In this thesis, we will examine the role of these signaling pathways in controlling cell fate of adult muscle progenitors.

**Embryonic muscle development**

Skeletal muscle is specified and patterned embryonically. Transient epithelial blocks of tissue that lie along the neural tube called somites arise from pre-somatic mesoderm in a process called somitogenesis (Christ and Ordahl, 1995, Tajbakhsh and Cossu, 1997). The maturing somite then undergoes an epithelial to mesenchymal transition to form the sclerotome which gives rise to cartilage and bone and the dermomyotome
which gives rise to dermis and all skeletal muscle (Christ et al., 1998, Kalcheim and Ben-Yair, 2005).

Skeletal muscle development is tightly controlled by temporal specific expression of key transcription factors. One important group is the $Pax$ family of genes, which are characterized by a DNA binding paired domain. PAX3 and its parologue PAX7 have overlapping functions for muscle specification (Buckingham, 2007). Loss of PAX3 embryonically leads to defects in progenitor cell migration (Franz et al., 1993). This is due to a loss of $c$-$met$ expression in $Pax3$ null mice leading to loss of activation of its ligand, HGF (hepatocyte growth factor, which is required to target myogenic progenitors (Epstein et al., 1996, Dietrich et al., 1999). Loss of PAX7 leads to normal skeletal muscle formation but with reduced satellite cell numbers (Mansouri et al., 1996, Oustanina et al., 2004). In the absence of both PAX3 and PAX7, only the embryonic muscle of the myotome is formed and all further muscle development is arrested (Relaix et al., 2005). Thus, PAX3 and PAX7 play an important role in early specification process of muscle development. As muscle continues to differentiate, $Pax$ gene expression is lost (Goulding et al., 1994).

Continued myogenesis requires signaling from morphogens such as sonic hedgehog (Shh) from the notochord and Wnts from the neural tube to the somites (Münsterberg and Lassar, 1995, Gustafsson et al., 2002), resulting in expression of members of the MyoD family of basic-helix-loop-helix (bHLH) transcription factors, specifically, MYOD, MYF5
and Myogenin (MYOG) (Arnold and Braun, 2000). Both Pax3 and Pax7 can also induce expression of Myf5 and MyoD (Maroto et al., 1997, Tajbakhsh et al., 1997). The basic domain of these proteins bind DNA and helix-loop-helix domain allows protein dimerization to regulate transcriptional activity (Maroto et al., 2008). These muscle specific transcription factors bind to conserved “E-box” (CANNTG) sequences that are found in the promoters of important muscle specific genes (Molkentin and Olson, 1996, Berkes and Tapscott, 2005, Blais et al., 2005). Null mutants of MyoD or Myf5 alone can successfully form skeletal muscle (Rudnicki et al., 1992, Braun et al., 1992). However, the MyoD and Myf5 double knockout lacks skeletal muscle (Rudnicki et al., 1993). Loss of Myog leads to severe reduction of differentiated skeletal muscle due to arrested myoblast differentiation (Hasty et al., 1993, Rawls et al., 1995). Thus, MYOD and MYF5 are important determiners of myogenesis while MYOG is important for continued differentiation along the myogenic path. The importance of MyoD family of transcription factors in specifying myogenic fates is illustrated by the fact that their ectopic expression can induce myogenic differentiation in many non-muscle cell types (Davis et al., 1987, Choi et al., 1990).

Satellite cells have been shown to arise from the dermomyotomal compartment of the somite (Kassar-Duchossoy et al., 2005, Schienda et al., 2006, Lepper and Fan, 2010). PAX7 expression in myogenic progenitors at E12.5 specify these cells as satellite cells (Lepper and Fan,
As fetal muscle development continues, satellite cells are enveloped under the basal lamina that forms around new muscle fibers, thus localizing them to the stem cell niche wherein they are found in adult musculature (Seale et al., 2000, Relaix et al., 2005, Broek et al., 2010).

**Adult skeletal muscle and satellite cell mediated repair**

Muscle fibers are the basic contractile unit of skeletal muscle. Myofibers are multi-nucleated, elongated, terminally differentiated and surrounded by a connective tissue layer. Groups of myofibers form bundles that are innvervated by motor neurons and express characteristic structural molecules such as Myosin Heavy Chain (MHC). Movement of muscle is facilitated by myotendinous junctions at the end myofiber bundles that allow attachment to the skeletal framework.

Adult skeletal muscle is prone to mechanical damage after intense physical activity or due to aging (Kirkendall and Garrett, 1998). Damage can also be due to indirect causes such as neurological dysfunction or genetic disease such as muscular dystrophies (Mercuri et al., 2002, Mendell et al., 2006). Although damaged muscle can repair itself, complete loss of muscle groups is irreversible; mammals cannot replace lost muscle groups (Standish and Eversole, 1970). In adult skeletal muscle, satellite cells remain quiescent and have characteristically shrunken cytoplasm and abundant heterochromatin in the nucleus.
(Schultz et al., 1978), reflecting their transcriptional and mitotic inactivity. Expression of certain key genes is associated with quiescence in satellite cells, such as Nestin (Day et al., 2007) and Msx-1 (Cornelison et al., 2000) (Figure 2).
Satellite cells express specific markers as they progress along the myogenic differentiation pathway. Quiescent satellite cells express markers such as Nestin, Mstn and Msx-1 along with Pax7. As they become activated, they express structural genes such as Desmin and M-cadherin. They also start to express the myogenic bHLHs, MyoD and Myf5. NotchICD is also detected in these cells. As satellite cells continue to differentiate, they become committed myoblasts and express the late myogenic marker Myog. Continued differentiation results in morphological changes as myoblasts elongate, form myocytes and begin to express structural genes such as MHC. Myocytes eventually fuse together to form a multi-nucleated myofiber.
When muscle is injured, damaged muscle fibers release cytokines such as CCL3, CCL8 and CCL7 that lead to an influx of macrophages and neutrophiles (McLoughlin et al., 2003, Warren et al., 2005, Zhang et al., 2009). These cytokines contribute to the activation of PAX7 positive satellite cells, reentry into the cell cycle and subsequent proliferation (Merly et al., 1999, Tidball and Villalta, 2010, Sambasivan et al., 2011). As satellite cells continue to differentiate and form myocytes, they begin to express muscle specific structural genes such as MHC (Sacks et al., 2003) thus contributing to the healing myofiber (Collins et al., 2005) (Figure 2).

Multiple signaling molecules play an important role in satellite cell function. MSTN, a secreted member of the TGF-β superfamily of signaling proteins, is an important regulator of satellite cell function. A loss of expression of MSTN leads to increase in muscle mass in mice, sheep, dogs, cattle and humans (McPherron and Lee, 1997, Kambadur et al., 1997, Lee and McPherron, 2001, Schuelke et al., 2004, Clop et al., 2006, Mosher et al., 2007). Loss of Mstn also leads to improved muscle healing after damage (McCroskery et al., 2005). Overexpression of Mstn in adult mice causes a wasting syndrome characterized by loss of muscle and adipose tissue (Zimmers et al., 2002). Signaling by MSTN induces phosphorylation of SMAD2/3, transcription factors that are effectors of TGF-β signaling in MSTN dependent satellite cell response (Langley et al., 2002, Rebbapragada et al., 2003, Sartori et al., 2009).
Active Notch signaling has been shown to repress differentiation in myoblasts and C2C12s by inhibiting MyoD expression (Kuroda et al., 1999, Nofziger et al., 1999, Wilson-Rawls et al., 1999, Buas et al., 2009). Conversely, inhibiting active Notch signaling has been shown to enhance differentiation of myoblasts (Kitzmann et al., 2006). Also, a conditional knockout of RBP-Jk or a hypomorphic DLL1 allele result in improper myogenic differentiation with a loss of Pax3/Pax7 positive satellite cells (Vasyutina et al., 2007, Schuster-Gossler et al., 2007). Notch1 is expressed in quiescent satellite cells however, Notch1ICD is only detected in activated proliferating satellite cells (Conboy and Rando, 2002). The reduced regenerative capacity of aged muscle is attributed to loss of DLL1 ligand, leading to loss of active Notch signaling (Conboy et al., 2003). Recent work has also shown that active Notch signaling is required for the maintenance of a quiescence in satellite cells (Bjornson et al., 2011). Taken together, these data suggest that active Notch signaling is important for maintenance of a progenitor pool of satellite cells in muscle.

Cross talk between Notch and Wnt pathways has also been shown to be important for satellite cell differentiation. In the absence of Wnt signaling, GSK3β inactivates the effector transcription factor involved in Wnt signaling, β-catenin. When canonical Wnt signaling takes places, GSK-3β is inactivated allowing β-catenin translocation into the nucleus and target gene expression. Work by Brack et al. showed that high amounts of Notch signaling in satellite cells correspond to active GSK-3β
leading to low Wnt signaling. However, as satellite cells continue to differentiate, GSK-3β is inactivated leading to increased Wnt signaling and a corresponding decrease in Notch activity (Conboy and Rando, 2002, Brack et al., 2008).

In satellite cells, Numb has been shown to be expressed in a biphasic manner, with expression detected in quiescent cells that is lost as cells become activated and then a return of expression as cells remain in culture for over 72 hours (Conboy and Rando, 2002). This expression pattern suggests that Numb might play an important role in controlling the switch between activation and proliferation in muscle progenitor cells.

**Muscle regeneration**

Mammalian muscle regeneration is restricted to the process of muscle repair that is carried out primarily through the action of satellite cells. Defects in the systems controlling muscle repair lead to muscular dystrophies, genetic disorders that cause muscle wasting (Mercuri et al., 2002). Mammalian muscle repair requires the presence of a scaffold where dividing satellite cells can intercalate to contribute to damaged myofibers. Additionally, true *de novo* regeneration of muscle and its associated tissues such as nerves, cartilage and bone is not observed in mammals. Amniote vertebrates, specifically some reptiles, can regenerate their tails and create new muscle as a part of this process (reviewed in
Understanding the molecular mechanisms that control this process has the potential to further cell-based regenerative medicine.

Organisms can regenerate by three possible mechanisms. Regeneration could take place by dedifferentiation of cells at the site of injury by formation of a blastema. Alternatively, morphallactic regeneration or tissue remodeling could occur, as is seen in planarians where anatomical structure and proportion is restored but growth is required to return to a normal size. The third possibility is the recruitment of stem cells to repopulate the damaged area and regenerate lost tissues. Stem cells could arise from each individual type of tissue that needs to be regenerated or one type of mesenchymal cell could be responsible for transdifferentiating into all required tissue types.

Satellite cells serve as a useful model system to study adult stem cell biology and are a promising candidate for cell therapies in muscle regeneration. A single normal mouse muscle myofiber has between 10 – 25 satellite cells associated with it (Collins et al., 2005). Indeed, injection of as few as 10 normal satellite cells into dystrophin-deficient mdx mouse muscle restores dystrophin expression in muscle fibers (Partridge et al., 1989). Satellite cells localize to a specific niche along the myofiber and express markers that allow for their detection and isolation. They can be grown and manipulated in vitro easily. Satellite cells have been identified and cultured from zebrafish, frogs, mice, rats, pigs and humans (Mauro, 1961, Shafiq and Gorycki, 1965, Schmalbruch and Hellhammer, 1975,
Anamniote vertebrates such as fish and amphibians can regenerate limbs, fins and tails. Amniote vertebrates such as reptiles and birds have reduced regenerative capacity but reptiles can regenerate tail appendages (Tsonis, 2000). Specifically, one group of reptiles, the anole lizards, such as *A. carolinensis*, can self-amputate or autotomize their tails along vertebral break points when danger is perceived and then completely regenerate nerve, skin, cartilage and skeletal muscle. Although regeneration has been documented in other reptiles, such as regeneration of the tail and jaws of crocodilians and the shells of turtles (Carlson, 2007), among the amniotes, lizards show the most regenerative capacity.

After tail autotomy, the lizard has to completely regenerate tissues of a variety of lineages. Since the anatomical structure of the newly regenerated tail is markedly different from the original tail (Cox, 1968a), true morphallactic regeneration, where structural regeneration takes place with very little cell proliferation (Saló and Baguñà, 2002), seems unlikely. This suggests that a mesenchymal cell population might be involved in the regeneration process. There are three potential sources of cells for the regenerating tail. New cells could be derived from dedifferentiated muscle cells that would re-enter the cell cycle. This has been demonstrated in urodeles such as axolotl (Lo et al., 1993, Echeverri et al., 2001). Circulating non-hematopoietic mesenchymal stem cells specific to bone...
marrow, Bone Marrow Stromal Cells (BMSCs) could provide a source of cells for various somatic lineages (Dezawa et al., 2005). Satellite cells represent another possible source of cells and under specific culture conditions have been induced to differentiate into adipogenic, osteogenic and chondrogenic lineage in mammals (Wada et al., 2002, Morrison et al., 2010). It is currently unknown whether satellite cells in adult *A. carolinensis* muscle provide a source of cells for the regenerating tail.

Functional muscle repair and regeneration depend on tightly controlled genetic and signaling events in satellite cells. This thesis will examine the function of Numb, an important satellite cell fate determinant, during the skeletal muscle repair process. Additionally, this thesis will determine if satellite cells from an amniote with high regenerative capacity, *A. carolinensis* could contribute to the enhanced capacity of this species.
CHAPTER 2

ROLE OF NUMB IN SATELLITE CELL MEDIATED MUSCLE REPAIR

Introduction

Adult skeletal muscle consists of myofibers that have a continuous cytoplasm and are multinucleated. In the adult, normal muscle functions such as strenuous physical activity results in muscle damage. In muscular dystrophies, where adult muscle is incapable of healing itself, over time these injuries lead to irreplaceable muscle loss and wasting (Hoffman et al., 1987).

Satellite cells are an adult stem cell population necessary for repair of postnatal muscle (Chargé and Rudnicki, 2004, Karalaki et al., 2009). When muscle is injured, quiescent satellite cells become activated and undergo proliferative expansion. A subset of this activated population then returns to a quiescent state to repopulate the healed myofiber with satellite cells for future rounds of damage. Thus, satellite cells have to make a binary fate decision after activation between differentiating or returning to quiescence (Figure 1). The molecular processes involved in this cell fate decision process remain unclear.

Notch signaling is an evolutionarily conserved signaling pathway that regulates cell fate decisions of progenitor cells (Bray, 2006). Upon ligand binding, the Notch receptor undergoes a series of proteolytic cleavages that result in the formation of the Notch intracellular domain
(NotchICD), which translocates into the nucleus to form a complex with transcriptional activators that are members of the CSL family of transcription factors, including RBP-Jk (Kopan and Ilagan, 2009). Previous work has demonstrated the importance of Notch signaling in satellite cell function. Notch1 is expressed in quiescent satellite cells however, Notch1ICD is only detected in activated proliferating satellite cells (Conboy and Rando, 2002). Inhibition of active Notch signaling results in enhanced differentiation in human and mouse myoblasts (Kitzmann et al., 2006) and active Notch signaling represses differentiation in C2C12s (Kuroda et al., 1999). Additionally, inhibition of Notch signaling during the satellite cell proliferation phase of muscle repair results in an increase in muscle progenitor cells (Brack et al., 2008).

Important modifiers of Notch signaling have been shown to play a role in satellite cell biology. The cytoplasmic protein NUMB binds to the intracellular domain of Notch1 and targets it for ubiquitination by recruiting the E3 ubiquitin ligase, Itch thus inhibiting active Notch signaling (McGill and McGlade, 2003, Beres et al., 2011). Also, both Notch and Numb negatively regulate protein expression (Chapman et al., 2006). Numb has two important functional domains, the phosphotyrosine binding domain (PTB) and the proline rich region (PRR) domains. Isoforms of Numb are generated by alternative splicing of two exons that have been shown to varying functions (Dho et al., 1999, Verdi et al., 1999).
Previous experiments suggest that NUMB plays an important role in satellite cell function. Embryonically, overexpression of NUMB in the myotome compartment of the somite led to an increase in the muscle progenitor cell population (Jory et al., 2009), which matches with earlier observations that NUMB expression is limited to the developing myotome (Venters and Ordahl, 2005). In adult skeletal muscle, NUMB has been shown to localize asymmetrically in dividing satellite cells (Conboy and Rando, 2002, Shinin et al., 2006), suggesting that NUMB plays a role in satellite cell function. Conboy et al, 2002 showed that NUMB is present in quiescent satellite cells, expression is lost when cells become activated. When satellite cell cultures remain in culture for 72 hours, NUMB expression is reestablished. This suggests that NUMB might play a biphasic role in determining satellite cell fate; NUMB might be required to maintain a progenitor pool of cells early on and again later to suppress Notch to allow myogenic differentiation. Investigators have reported that in activated muscle progenitor cell populations, overexpression of Numb leads to increased differentiation (Conboy and Rando, 2002, Kitzmann et al., 2006). NUMB has also been shown to cosegregate with the self renewal marker, PAX7 during asymmetric divisions in satellite cells, suggesting that NUMB is important for a return to quiescence after activation in satellite cells (Shinin et al., 2006). Since effective satellite cell function is required for muscle repair, NUMB could play an important role in this repair process. In these studies, we examine the previously
uncharacterized function of NUMB in postnatal skeletal muscle repair and satellite cell proliferation and differentiation.

Results

Improper muscle repair in conditional *Numb* null mice

Traditional knock outs of *Numb* in mice are embryonically lethal at embryonic day (E)11.5 due to neural tube defects (Zhong et al., 2000). Knockout of the functionally related gene *Numblike* (*Nbl*) results in mice that are viable, fertile and exhibit no phenotypes (Petersen et al., 2002). Therefore, in order to examine the role of NUMB in adult muscle repair, mice with floxed alleles of both *Numb* and *Nbl*, *Numb*\textsuperscript{tm1Zili/tm1Zili} (\textit{Numb fl/fl}) and *Nbl*\textsuperscript{tm1Zili/tm1Zili} (\textit{Nbl fl/fl}) (Zilian et al., 2001) were used.

In order to induce recombination at the *Numb* and *Nbl* loci, transgenic mice with a ubiquitously expressed Cre recombinase CAGG ER-Cre\textsuperscript{TM}, (\textit{ER-Cre}) (Hayashi and McMahon, 2002) were used. In order to examine the effect that a loss of *Numb* and *Nbl* has on muscle repair, *Numb*\textsuperscript{fl/fl} *Nbl*\textsuperscript{fl/fl} mice were bred with \textit{ER-Cre} mice and injected with tamoxifen to induce excision at the floxed loci.

Skeletal muscle repair was induced by damaging the quadriceps femoris with cardiotoxin (Wallace and McNally, 2009). Since mouse muscle is completely healed 10 to 14 days after damage, we examined 7 and 10 days post cardiotoxin to evaluate the repair response (Figure 3).
Figure 3: Impaired regeneration in *Numb* deficient muscle

Cardiotoxin was injected in the left quadriceps femoris muscle. Muscle was harvested for histological analysis at 7 and 10 days. Left panels are H&E and right panels are trichrome stained (Pink: cytoplasm, Dark blue/purple: nuclei, Blue: collagen). Genotypes are as indicated at 7 and 10 days post-cardiotoxin. Scale bars are as indicated.
Muscle was harvested, fixed, embedded and sectioned for histological analysis. Muscle sections from \textit{Numb}^{fl/fl} \textit{Nbl}^{fl/fl} \textit{ER-Cre} and \textit{Numb}^{fl/fl} \textit{Nbl}^{+/+} \textit{ER-Cre} tamoxifen treated mice showed inflammatory and dystrophic responses characterized by an increased endomysial space, infiltration of fibroblasts, collagen deposition and degenerating myofibers. (Figure 3, panels A - H). Damaged muscle from tamoxifen treated \textit{Numb}^{+/+} \textit{Nbl}^{fl/fl} \textit{ER-Cre}, \textit{Numb}^{fl/+} \textit{Nbl}^{fl/+} \textit{ER-Cre} and \textit{Numb}^{fl/fl} \textit{Nbl}^{fl/fl} control mice did not show any repair defects (Figure 3, panels I – P).

\textbf{Numb and Numblike are knocked out in culture with tamoxifen treatment}

Culture of satellite cells \textit{in vitro} allows us to manipulate them and examine their functionality. Since \textit{Numb} deficient muscle has a repair defect, we isolated satellite cells from the quadriceps femoris muscles. The activated form of tamoxifen, 4-hydroxytamoxifen (4OH-T) was used to induce recombination at \textit{Numb} and \textit{Nbl} loci. As a control, cells were also treated with ethanol.
Figure 4: Isolation and genotyping of satellite cell lines

Cells were treated with 4-hydroxytamoxifen (4OH-T), or ethanol (EtOH). Cells were genotyped to determine the percent of *Numb* and *Numblike* loci that are excised.

A and B. Primers flanking downstream loxP sites on *Numb*\(^{fl/fl}\) and *Nbl*\(^{fl/fl}\) alleles were designed. PCR products were run on 3% agarose gel and loss of PCR product was used to confirm excision of *Numb* or *Nbl*. E: ethanol treated. T: 4OH-T treated. Upon Cre recombinase mediated excision, one loxP site is removed leading to a loss of PCR product.

C and D. Genomic QPCR was used to quantify the percentage of *Numb* loci that were excised. Control primers provide total genomic DNA input and samples are compared between \(\Delta\Delta C_t\) values for excised and unexcited primer sets to determine percent recombined loci. Only cell lines with >95% recombination at *Numb* locus were used for studies.
In order to determine if treatment with 4OH-T effectively induces recombination at the *Numb* and *Nbl* loci, primers were designed flanking the 3’ loxP sites of *Numb*<sup>fl/fl</sup> and *Nbl*<sup>fl/fl</sup> alleles (Figure 4a). For the *Numb* locus, the wildtype band generates a 379 bp product and the floxed allele a 413 bp product due to inclusion of the loxP site. For the *Numblike* locus, the wildtype allele produces a 269 bp product and the floxed allele a 303 bp product. Cells with the *ER-Cre* transgene and treated with 4OH-T will excise the floxed allele resulting in a loss of the larger product (Figure 4b). Analysis of PCR product on a 3% agarose gel demonstrates that 4OH-T treatment successfully induces recombination at *Numb* and *Nbl* loci (Figure 4b).

In order to quantify the percentage of excision mediated by *ER-Cre*, primers were created that flank the excised and unexcised *Numb* locus; control primers that lay outside the loxP sites were used as control for total *Numb* loci. QPCR on genomic DNA was carried out and ΔΔCt values for excised and unexcised primer sets was compared in each cell type to quantify percentage recombination at the *Numb* locus (Figure 4c). Treatment of satellite cell lines with 4OH-T led to successful excision at the *Numb* and *Nbl* loci. *Numb*<sup>fl/fl</sup>*Nbl*<sup>fl/fl</sup> *ER-Cre* and *Numb*<sup>fl/fl</sup>*Nbl*<sup>+/+</sup> *ER-Cre* satellite cells show greater than 95% recombination at the floxed allele (d) and thus were used for further analysis. *Numb*<sup>fl/+</sup>*Nbl*<sup>fl/+</sup> *ER-Cre* satellite cells show ~50% recombination of the *Numb* locus, due to one wildtype *Numb* allele in these cells (Figure 4d).
Loss of Numb leads to hypotrophic myotubes

Differentiating satellite cells progress along the myogenic differentiation pathway and fuse with myotubes. Impaired repair seen in conditional Numb null mice (Figure 3) could be due to an inability of satellite cells without NUMB to effectively progress down the myogenic pathway to heal the damaged myofiber. In order to evaluate the differentiation ability of these cells, we cultured them in low serum conditions. Numb\textsuperscript{fl/fl} Nbl\textsuperscript{fl/fl} ER-Cre 4OH-T treated satellite cells formed myotubes that had fewer nuclei, did not branch extensively and did not stain as robustly for MHC (Figure 5, a-d). Additionally, in order to characterize the differentiation process, myotubes were harvested every 12 hours. Whole cell lysate was prepared from cells at every time point and 5µg of lysate was run on an SDS-PAGE gel. MHC protein was detected on western blots. Numb\textsuperscript{fl/fl} Nbl\textsuperscript{fl/fl} ER-Cre 4OH-T treated cells show delayed expression of MHC as compared to ethanol treated controls indicating that Numb deficient satellite cells undergo less effective differentiation (Figure 5d).
Figure 5: Differentiation of Numb deficient satellite cells

A-D: Satellite cells were treated with either EtOH or 4OH-T for 48 hours, and cultured in differentiation medium for 60 hours. IHC was done with an anti-MHC specific antibody and DAB colorimetric detection. Panels are as labeled. Numb\textsuperscript{fl/fl} Nb\textsuperscript{fl/fl} ER-Cre\textsuperscript{Tg/Tg} 4OH-T treated cells (panel D) formed myotubes that had fewer nuclei, were smaller in diameter, and did not stain as robustly for MHC.

E. Western blot of total protein lysate from Numb\textsuperscript{fl/fl} Nb\textsuperscript{fl/fl} ER-Cre myotubes. Lanes were loaded with 5µg of protein and are as labeled. Anti-MHC antibody was used to detect the 210 kD protein on blots. Myotubes harvested from Numb\textsuperscript{fl/fl} Nb\textsuperscript{fl/fl} ER-Cre 4OH-T treated satellite cells demonstrate a delay in MHC expression compared to those that were ethanol treated.
Conditional *Numb* knockout satellite cells have a proliferation defect

Functional skeletal muscle repair requires a controlled proliferative expansion of satellite cells before they can fuse with the damaged myofiber. Reduced healing capacity of *Numb* deficient muscle might be caused by defective satellite cell function. Therefore, *Numb* deficient satellite cells were analyzed for changes in population growth. Normal proliferation rates were observed for *ER-Cre* ethanol and 4OH-T treated cells as compared to control *Numb*^fl/fl^ *Nbl*^fl/fl^ ethanol and 4OH-T treated cells, indicating that Cre recombinase does not deleteriously affect proliferation. However, *Numb*^fl/fl^ *Nbl*^fl/fl^ *ER-Cre* 4OH-T treated cells had significantly lower cell numbers per day as compared to *Numb*^fl/fl^ *Nbl*^fl/fl^ 4OH-T treated controls (Figure 6). *Numb*^fl/fl^ *Nbl*^+/−^ *ER-Cre* 4OH-T treated cells also showed decreased proliferation. These data demonstrate that loss of *Numb* leads to loss of proliferation in satellite cells.

Conditional *Numb* knockout satellite cells do not show increased levels of apoptosis

Decreased satellite cell numbers in culture could be due to increased apoptosis. In order to test this, a luciferase assay that detects Caspase3/7 activity, markers for cellular apoptosis, was carried out on *Numb*^fl/fl^ *Nbl*^fl/fl^ *ER-Cre* and *Numb*^fl/fl^ *Nbl*^fl/fl^ ethanol and 4OH-T treated
cells (Figure 7). There is no significant difference in luciferase activity between any of the cell lines tested. These results showed that reduced cell numbers in $\text{Numb}^{\text{fl/fl}} \text{Nbl}^{\text{fl/fl}} \text{ER-Cre} \ 4\text{OH-T}$ treated cells were not due to apoptosis.
Figure 6: *Numb* deficient satellite cells do not proliferate
Satellite cells were plated at 1X10^5 cells/well in growth medium in triplicate. Cells were trypsinized daily and stained with 0.4% trypan blue and counted. *Numb^fl/fl* Nbl^fl/fl^ ER-Cre-treated cells have a static cell number over time indicating that these cells are not proliferating as compared to *Numb^fl/fl* Nbl^fl/fl^ ethanol (EtOH) treated controls. These data demonstrate that loss of *Numb* leads to a significant decrease (p<0.01) in satellite cell proliferation by day 4. Data are mean numbers of cells +/- s.d. and significance was measured by one-way ANOVA.
Satellite cells were plated at 1x10^5 cells/well in triplicate. A Caspase-Glo 3/7 Assay was used to quantify apoptotic activity in cells over a 48 hour period. *Numb* deficient satellite cells do not have increased levels of apoptosis in culture (purple bars). No significant difference in the overall apoptosis levels was detected between satellite cells that have been treated with either ethanol or 4OH-T over 48 hours. Data are average luciferase levels +/-s.d. from two duplicate experiments for each timepoint.
<table>
<thead>
<tr>
<th>Gene</th>
<th>$N_{b}^{fl/fl}N_{bl}^{fl/fl}ER-Cre$ cells treated with 4OH-T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Notch1</td>
<td>$0.80+/-.15; p=0.318174$</td>
</tr>
<tr>
<td>Deltalike1</td>
<td>$0.82+/-.12; p=0.198402$</td>
</tr>
<tr>
<td>Hes1</td>
<td>$1.16+/-.07; p&lt;0.05$</td>
</tr>
<tr>
<td>Hey2</td>
<td>$1.28+/-.35; p=0.248351$</td>
</tr>
<tr>
<td>Hes6</td>
<td>$4.88+/-.08; p&lt;0.0001$</td>
</tr>
<tr>
<td>Hes3</td>
<td>$5.74+/-.346; p=0.079378$</td>
</tr>
<tr>
<td>Pax7</td>
<td>$0.96+/-.78; p=0.656296$</td>
</tr>
<tr>
<td>CD34</td>
<td>$1.59+/-.25; p&lt;0.05$</td>
</tr>
<tr>
<td>Myod1</td>
<td>$0.85+/-.01; p=0.573410$</td>
</tr>
<tr>
<td>myostatin</td>
<td>$71.56+/-.23.44; p&lt;0.05$</td>
</tr>
<tr>
<td>P21</td>
<td>$199.04+/-.58.93; p&lt;0.05$</td>
</tr>
</tbody>
</table>

Table 1: Candidate gene QRT-PCR on $Numb$ deficient satellite cells

$Numb^{fl/fl}N_{bl}^{fl/fl}ER-Cre$ satellite cells were treated with 4OH-T or ethanol for 48 hours, then cultured for 4 days. Total RNA was harvested and cDNA was synthesized. Using gene specific primers that span an intron, QRT-PCR was done using Sybrgreen. $\Delta\Delta Ct$ method for calculating relative gene expression was used. Relative gene expression was calculated in this dataset using $Numb^{fl/fl}N_{bl}^{fl/fl}ER-Cre$ cells treated with ethanol as control. Statistical analysis was done using one-way ANOVA with confirmation of significance by the post-hoc Tukey test. Significant relative gene expression values are indicated in green.
**Mstn and p21 are up-regulated**

Loss of proliferation of *Numb* deficient satellite cells could be irregularities in gene expression in these cells. Expression level of candidate genes important for satellite cell function was examined (Table 1). QRT-PCR analysis of *Numb*<sup>fl/fl</sup> *Nbl*<sup>fl/fl</sup> *ER-Cre* 4OH-T treated satellite cells showed that there is no significant change in the markers of satellite cells such as Pax7 and MyoD. Expression of Notch receptor and ligandDll1 also remain unchanged. However, the downstream targets of Notch signaling, Hes1 and Hes6 show a slight increase indicating that Notch signaling might be active in these cells.

Interestingly, the cell cycle regulators *Mstn* and *p21* are significantly up-regulated in these cells (Table 1). MSTN is an important member of TGF-β superfamily and is well studied for its role in regulating adult skeletal muscle mass, as evidenced by the increase in muscle mass phenotype observed when *Mstn* expression is lost in cows, pigs and humans (Schuelke et al., 2004). MSTN acts by inducing expression of *p21* leading to an arrest of myoblasts in the G<sub>1</sub> phase of the cell cycle (Thomas et al., 2000).

Lack of proliferation in *Numb* deficient satellite cells might be due to increased *Mstn* expression. In order to test *Mstn* is responsible for the proliferation defect observed in these cells, we transfected cells with siRNA specific for *Mstn* (Figure 8a). *Mstn* expression was knocked down
by ~90% in Numb\textsuperscript{fl/fl} Nbl\textsuperscript{fl/fl} ER-Cre cells after siRNA treatment. Inhibition of the growth inhibitor Mstn leads to improved proliferation rates, as evidenced by more Numb\textsuperscript{fl/fl} Nbl\textsuperscript{fl/fl} ER-Cre ethanol and Mstn specific siRNA treated cells per day as compared to Numb\textsuperscript{fl/fl} Nbl\textsuperscript{fl/fl} ER-Cre ethanol and scramble siRNA treated cells (b). Treatment of Numb\textsuperscript{fl/fl} Nbl\textsuperscript{fl/fl} ER-Cre 4OH-T treated satellite cells with Mstn specific siRNA partially rescued the proliferation defect as compared to the control Numb\textsuperscript{fl/fl} Nbl\textsuperscript{fl/fl} ER-Cre 4OH-T and scramble siRNA treated cells (Figure 8b). These data indicate that up-regulation of Mstn results in proliferation defect in Numb deficient satellite cells.
Figure 8: RNAi knockdown of Mstn rescues proliferation in Numb deficient satellite cells

A. *Numb*^{fl/fl} *Nbl*^{fl/fl} ER-Cre satellite cells that have been 4OH-T or ethanol treated were transfected with Mstn or scramble RNAi. cDNA was quantified using Mstn specific, intron spanning primers and QRT-PCR was done with Sybrgreen. All samples were normalized to Gapdh transcript using ΔΔCt analysis and expressed as relative gene expression. Percent knockdown of Mstn is indicated above RNAi treated samples.

B: Proliferation assay to determine if Mstn knockdown rescues proliferation defect. *Numb*^{fl/fl} *Nbl*^{fl/fl} ER-Cre 4OH-T or ethanol treated satellite cells were plated at 1x10^5 cells/well at day 0. Cells were trypsinized, stained with 0.4% trypan blue and triplicate wells were counted daily. Mstn knockdown results in a rescue of the proliferation defect in Numb deficient satellite cells and increased the proliferation rate of ethanol treated cells. These data are the result of triplicate wells from two separate experiments. Lines are as indicated.
Discussion

The ability of adult muscle to repair and regenerate itself after injury depends on successful satellite cell function. In response to damaged myofibers, satellite cells become activated, proliferate and then have to make a binary cell fate decision to differentiate or return to quiescence. NUMB, a cell fate determinant, asymmetrically segregates in proliferating satellite cell populations; this suggests a role for this protein in satellite cell function in adult muscle. Conditional loss of Numb from satellite cells mediated by a tamoxifen inducible Cre recombinase was used to determine its function in satellite cell mediated muscle repair. Numb deficient muscle is unable to repair effectively (Figure 3). Isolation of satellite cells from Numb^{fl/fl} Nbl^{fl/fl} ER-Cre mice and subsequent treatment with 4OH-T allowed us to directly determine if a failure of proper muscle repair in Numb null mice is due to defects in the activated satellite cell population (Figure 4). A lack of Numb leads to decrease in satellite cells proliferation (Figure 6). Conditional Numb null satellite cells also up-regulate the muscle growth factor Mstn (Table 1) and the proliferation defect was partially rescued by Mstn siRNA treatment (Figure 8b), suggesting that the loss of proliferation is caused by Mstn misregulation in these cells.
Notch signaling is an important regulator of satellite cell function. Specifically, conditional knockout of RBP-Jκ or a hypomorphicDll1 allele resulted in improper myogenic differentiation with a loss of Pax3/Pax7 positive satellite cells (Schuster-Gossler et al., 2007). Loss of Notch signaling leads to improved differentiation of myotubes (Kitzmann et al., 2006). Additionally, loss of RBP-Jκ, i.e. loss of Notch signaling, leads to capricious activation and differentiation without proliferation of the satellite cell population leading to satellite cell loss (Bjornson et al., 2011). Ectopic Notch1ICD expression in satellite cells also promoted proliferation and repressed myogenic differentiation (Conboy and Rando, 2002). Thus, active Notch signaling is important for maintenance of quiescence and subsequent proliferation of satellite cells. If the role of NUMB in satellite cell function was to inhibit Notch signaling in activated cells, one might expect to see an increase in number of progenitor cells, because Notch signaling should be increased due to loss of an inhibitor. However, we observe a decrease in proliferation rates of satellite cells without NUMB. NUMB has been shown to have a biphasic expression pattern in muscle precursor cell populations; expression is seen in quiescent satellite cell populations, is lost as these cells become activated and returns as cells are established in culture (Conboy and Rando, 2002). The unexpected loss of proliferation in Numb knockout satellite cells suggests a previously uncharacterized role for NUMB in mouse satellite cell function.
We also report the novel finding that loss of *Numb* in satellite cells resulted in an up-regulation *Mstn* expression. *Mstn* inhibits myoblast proliferation (Thomas et al., 2000) (Langley et al., 2002). In satellite cells, MSTN expression promotes quiescence and negatively regulates the self-renewal process by inhibiting progression of cells into S phase of cell cycle (McCroskery et al., 2003). *Mstn* expression in myoblast cells leads to increased differentiation (Manceau et al., 2008). Interestingly, MSTN has been shown to regulate myoblast differentiation through Notch signaling, indicating some cross talk between these pathways (McFarlane et al., 2011). Previous work has also shown that Notch and SMAD3, the effector transcription factor activated by MSTN signaling, antagonize each other in satellite cells such that active Notch signaling blocks SMAD3-dependent induction of cell cycle inhibitors such as p21 (Carlson et al., 2008). *Smad3*−/− mice show decreased satellite cell numbers, reduced capacity for healing and upregulate *Mstn* expression (Ge et al., 2011). It is possible that in the absence of NUMB, levels of Notch and SMAD3 protein change, causing misexpression of *Mstn* in these cells. These data suggest that

Thus, these data suggest a novel function for NUMB *in vivo* muscle repair mediated by satellite cell proliferation and differentiation by inhibition of components of the TGF-β signaling pathway.
Materials and Methods

Mouse lines, injury and histomorphometrics:

Mice with the conditionally floxed alleles $Numb^{tm1Zili/tm1Zili/J}$, referred to as $Numb^{fl/fl}$ and $Numbl^{tm1Zili/tm1Zili/J}$, referred to as $Nbl^{fl/fl}$ (Zilian et al., 2001) were obtained from Jackson labs. A tamoxifen inducible Cre recombinase expressing mouse, CAGG ER-Cre$^{\text{TM}}$, referred to as ER-Cre was crossed with the floxed alleles (Hayashi and McMahon, 2002). Adult (60 day old) mice of various genotypes were injected with 0.4mg tamoxifen (Sigma) dissolved in corn oil twice a day for 5 days. After allowing the mice to recover from injections for 28 days, the quadriceps muscle was injected with 100µl of 10µM cardiotoxin (Calbiochem, Darmstadt, Germany) with 0.2% India ink suspension to mark injection site. All protocols were approved by the Institutional Animal Care and and Research Advisory Committee and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Muscle was harvested at 7 or 10 day post cardiotoxin injection and fixed, embedded in paraffin, sectioned at 5µm and stained with hematoxylin and eosin or Gomorri’s one step trichrome stain. Transverse sections of muscle were photographed at 250x and a minimum of 500 myofibers was examined per animal. Longest diameters of healing myofibers were measured using Image Pro Plus v5.0 software.
(Cybernetics, Bethesda, MD). Statistical analysis was carried out using one way ANOVA and significance was defined as $p \leq 0.05$.

Satellite cell isolation and culture:

Satellite cells were isolated from 2-3 month old mice as previously described in (Allen et al., 1997). Briefly, hind limb muscle was excised, trimmed of fat and connective tissue, finely minced to a pulp, digested with 1.25mg of protease XIV (Sigma) for one hour at $37^\circ$C, triturated to extract satellite cells and passed through a 100µm nylon mesh. The cell suspension was spun down at 1500 x g for 10 mins and preplated in DMEM, 2% HS and 100µg/ml primocin (Invivogen, San Diego, CA) to remove fibroblasts and other debris for 2 hours. Satellite cells were grown on matrigel (BD Biosciences, San Diego, CA) in Hams F-10, 20% FBS, 10ng/ml bFGF (BD Biosciences) and 100µg/ml primocin. To induce recombination in floxed alleles, satellite cells in growth media were treated with 1µM 4OH-T (4-hydroxy tamoxifen) (Sigma) for 48 hours.

Differentiation and Proliferation Assays:

For differentiation assays, satellite cells were allowed to reach >85% confluency and switched to differentiation media (DMEM, 2% HS, 100µg/ml primocin) every day for 3 days or until large, fused myotubes were observed.
For proliferation assays, satellite cells were plated in matrigel coated 96-well plates at 1 x 10^5 cells/well at day 0. Cells were trypsized, stained with 0.4% trypan blue (CellGro, Manassas, VA) to exclude dead cells and counted using a hemocytometer every day for 6 days (or until fully confluent).

Apoptosis assay:

Satellite cells were plated at 1 x 10^5 cells/well in matrigel coated 96 well plates. Cells were lysed and caspase activity was measured using the Caspase-Glo 3/7 reagent (Promega, Madison, WI) and luciferase activity was measured after 30 mins incubation in white 96-well plates, using an FLx800 microplate reader (BioTek Instruments, Inc, Winooski, VT). Sample variables were performed in triplicate per experiment.

Quantitative RT PCR:

Proliferating satellite cells were lysed in Trizol (Invitrogen) for RNA isolation. RNA was treated with DNase I prior to cDNA synthesis using SuperScriptIII reverse transcriptase (Invitrogen, Grand Island, NY). cDNA was then quantified using transcript specific, intron spanning primers and real time PCR with Sybrgreen (Eurogentec, Fremont, CA). All samples were normalized to GapDH transcript using ΔΔCt analysis and expressed as relative amount of gene expression as compared to Numb^{fl/fl} Nbl^{fl/fl} EtOH treated control cell line.
siRNA mediated knockdown assay:

Satellite cells were seeded at $1 \times 10^5$ cells/ml in a 24 well plate. 24 hours after plating, 3 different Mstn specific siRNAs (Invitrogen) or scramble siRNA (Invitrogen) at a final total concentration of 50nM per well were transfected with 1.5µl per well of Lipofectamine RNAiMax (Invitrogen). Cells were either harvested for RNA or counted using a hemocytometer for 3 days.

Genomic Quantitative PCR:

DNA was purified from the appropriate cell line using a standard phenol chloroform extraction protocol. Purified DNA from all samples was used at final concentration of 25ng/µl. Control genomic primers, excised Numb locus primers and unexcised Numb locus primers were used at a final concentration of 0.5µM each. Quantitative PCR was carried out using a 2x Sybrgreen mastermix (Eurogentec). Ct values for locus specific primers were corrected for input DNA by subtracting out control genomic Ct value. For every sample, data was represented as percentage of excised vs unexcised Numb locus upto a total of 100%.
CHAPTER 3
CHARACTERIZING SATELLITE CELLS IN THE REPTILE ANOLIS CAROLINENSIS

Introduction

Epimorphic regeneration is the process by which, after loss of an appendage, an organism undergoes proliferation, grows and functionally restores the damaged part (Bryant et al., 1981, Agrawal et al., 2010). In vertebrates, this process is tightly regulated to prevent uncontrolled proliferation (Cardoso et al., 1993, Bedelbaeva et al., 2010, Pajcini et al., 2010). Although regenerative capacities vary greatly within vertebrates, they share some common features (Stocum, 1995, Brockes and Kumar, 2005). These include an initial inflammatory response at the site of injury, increase in cell number and finally terminal differentiation of cells and normal patterning to replace lost tissues (Hay and Fischman, 1961, Lentz, 1969, Bryant and Endo, 2002). In general, within the vertebrate family, regenerative capacity is seen in almost all tailed amphibians (urodeles) and fish, to some extent in reptiles and least observed in birds and mammals (Neufeld and Mohammad, 2001, Galis et al., 2003, Poss et al., 2003, Mochii et al., 2007).

Regeneration has been extensively studied in anamniote vertebrates. For example, the urodele, *Ambystoma mexicanum* (the axolotl) regenerates its appendages by the formation of a growth zone and
dedifferentiated cells, known as a blastema (Stocum, 1968). However, it is of interest to study the reptilian regeneration process because this group is the only amniote vertebrate (reptiles, birds and mammals) that retain considerable regenerative capacity. Reptiles do not regenerate limbs but can successfully regenerate their tails (Clause and Capaldi, 2006). Reptilian tail regeneration is more complex than limb regeneration because the tail contains more varied tissue types as compared to the limbs, such as the spinal cord and sensory ganglia. Reptiles are able to spontaneously shed their tails in response to perceived danger, a process referred to as autotomy (Mcconnachie and Whiting, 2003, Bernado and Agosta, 2005). The regenerated tail consists of an ependymal cell tube surrounded by a cartilaginous tube surrounded by musculature, fascia and skin (Alibardi et al., 1988, Alibardi, 1995a). A cut tail does not regenerate as effectively as an autotomized one, suggesting that the physiological events taking place during autotomy are important for functional regeneration (Jamison, 1964).

The green anole, *Anolis carolinensis*, autotomizes its tail and has been extensively studied (Tassava and Goss, 1966, Maderson and Licht, 1968, Egar et al., 1970, Alibardi, 1995a, Scehnet, 2006). The molecular processes involved in this regenerative process remain unclear. Morphological observations suggest that the anole tail does not form a blastema upon autotomy (Alibardi, 2010). Therefore, it is likely that regeneration of lost tissue requires a source of cells to form the new tail.
Several possibilities for such a mesenchymal source of cells exist. Cells could dedifferentiate from existing tissue, mesenchymal cells could migrate to the site of injury such as BMSCs or local populations of progenitor cells such as satellite cells could contribute to the regenerating tail.

The muscle specific stem cell, satellite cells represent a mesenchymal population that could contribute to the regenerating tail. Electron microscopy studies examining *A. carolinensis* muscle indicate the presence of satellite cells (Kahn and Simpson, 1974) and their incorporation into regenerating musculature (Alibardi, 1995b). Anole satellite cells have also been shown to differentiate into myotubes *in vitro* (Cox, 1968b, Chlebowski et al., 1973, Bayne and Simpson, 1975). However, there are no known adult progenitor cell populations to regenerate cartilage. This presents us with the problem of how *A. carolinensis* can successfully restore the functional cartilage tube seen in the regenerated tail.

Previous work has shown that mouse and human myogenic progenitor cells can be induced to undergo chondrogenic and osteogenic differentiation with extensive treatment with morphogens. For example, viral mediated transfection of BMP2 in C3H10T1/2 mesenchymal progenitor cells induces chondrogenic differentiation (Carlberg et al., 2001). Treatment with 100 ng/ml of BMP2 also induces osteogenic differentiation of the myoblast cell line, C2C12 (Katagiri et al., 1994).
Mammalian satellite cells have also been induced, under specific culture conditions, to differentiate into osteogenic and chondrogenic lineages (Asakura et al., 2001, Wada et al., 2002, Usas and Huard, 2007, Ozeki et al., 2007, Hashimoto et al., 2008, Morrison et al., 2010). These experiments involved treatment of satellite cells with large amounts (100-500 ng/ml) of BMP2, BMP4 or BMP7 or viral mediated transformation to stably express transcription factors in culture. However, inducing non-muscle differentiation pathways in these cells by administration of supraphysiological doses of morphogens does not accurately recapitulate the differentiation and patterning program that is carried out by regenerative organisms. Thus, in order to study the cellular and molecular mechanisms driving regeneration, a primary stem cell line from a regenerative organism such as *A. carolinensis* is required. In this work, we aim to characterize satellite cells isolated from *A. carolinensis* and examine their potential.

**Results**

**Characterization of satellite cells from *Anolis carolinensis***

*A. carolinensis* muscle is reported to contain satellite cells (Kahn and Simpson, 1974). With modifications to previously published protocols (Tatsumi et al., 2006), we isolated satellite cells from *A. carolinensis* limb
Figure 9: Satellite cells from *Anolis carolinensis* express Pax7
Satellite cells were plated on coverslips and allowed to grow overnight. Cells were fixed in methanol and stained with anti-PAX7 primary detected using FITC conjugated anti-mouse IgG. DAPI was used as a counterstain for nuclei. Images are at 200x magnification.

**A-B:** *A. carolinensis* satellite cells stained for PAX7 protein

**C-D:** No primary control *A. carolinensis* satellite cells

**E-F:** Mouse satellite cells stained for PAX7 protein
musculature. These cells demonstrated characteristic spindle shaped morphology in culture. In order to determine the molecular identity of cells isolated from *A. carolinensis*, we used the satellite cell marker PAX7, which has been shown to detect satellite cells in culture (Zammit, 2008). Anole cells were grown on coverslips, fixed and stained with anti-PAX7 antibody and detected using a FITC conjugated anti-mouse secondary (Figure 9, panels A and B). DAPI was used to visualize nuclei. A lack of FITC in the no primary antibody control (Figure 9, panels C and D) demonstrates that the FITC signal is specific. Mouse satellite cells (Figure 9, panels E and F) were used as the positive control and stain positively for PAX7. This is indicative of successful isolation and culture of satellite cells from a regenerative non-avian reptile.

In order to confirm that cells isolated from *A. carolinensis* are satellite cells, the newly published genome (Alföldi et al., 2011) was used to design quantitative RT-PCR assays. Since satellite cell and markers have been well studied in mouse (Chargé and Rudnicki, 2004), satellite cells isolated from CD-1 mice were used as positive controls. The mouse satellite cells used in these studies were from an outbred strain of mice (CD-1 mice) and are genetically variable (Chia et al., 2005). Therefore, these cells are an appropriate control for comparison with satellite cells from wild-caught *A. carolinensis*. QRT-PCR analysis showed that anole satellite cells express satellite cell markers such as *Pax3*, *Pax7* and *CD34*. The myogenic bHLHs, *MyoD* and *Myf5* were detected (Table 2). Structural
genes specific for satellite cells, *Desmin* and *M-cadherin* were also detected.

Anole and mouse satellite cells might have different growth dynamics in culture. In order to further characterize these satellite cells, 1 x 10⁴ cells were plated in triplicate in 96-well plated and counted daily. Comparison of growth rates of mouse and anole satellite cells demonstrated that these populations double within a similar time frame (Figure 10).
Table 2: Candidate gene QPCR for satellite cell markers
Using the newly published genome for *A. carolinensis* candidate gene QPCR was carried out on markers of satellite cells. Mouse satellite cells are used as a comparison. Markers such as *Pax3*, *Pax7* and *CD34* were detected in anole satellite cells. Muscle regulatory genes, *Myf5* and *MyoD* are also detected in both cell types. Additionally, satellite cell specific structural genes *Desmin* and *M-cadherin* are also detected.
Figure 10: Proliferation rates of anole and mouse satellite cells
Anole and mouse satellite cells were plated at $2 \times 10^4$ cells/well at day 0. Cells were trypsinized, stained with 0.4% trypan blue to exclude dead cells and counted daily for 6 days. Data represents mean of triplicates +/- s.d for each time point for each cell type.

![Graph showing proliferation rates of anole and mouse satellite cells.](image_url)
Since satellite cells should be able to form muscle, we challenged the cells isolated from *A. carolinensis* to differentiate in culture under low serum conditions to evaluate whether reptilian satellite cells can be induced to differentiate like their mammalian counterparts (Figure 11). *A. carolinensis* satellite cells formed multinucleated syncytia and expressed MHC as determined by IHC. Taken together, these data confirm that the anole cell population isolated are satellite cells and form myotubes.

**Anole satellite cells express chondrogenic and osteogenic genes in culture**

As described in the introduction, mammalian satellite cells have been shown to differentiate into various tissue lineages with high concentrations of morphogenic proteins, such as BMP2, or transfection with transcription factors such as *Runx2*. However, satellite cells isolated from mice do not express chondrogenic or osteogenic markers when cultured with differentiation medium that does not stimulate with morphogens such as BMP2 (Wada et al., 2002). In order to evaluate if anole satellite cells might play a role in the regeneration process, they were cultured in the absence of these morphogens to examine their endogenous potential for plasticity. Anole and mouse satellite cells were plated in triplicate at high confluency (>90%) and cultured in chondrogenic medium.
Figure 11: Differentiation of mouse and anole satellite cells

(A) Mouse and (B) anole satellite cells were grown to high confluency and challenged to differentiate. After 60 hours in differentiation media, cells were fixed with methanol and stained for MHC using immunohistochemical staining and detected using DAB substrate. Hemotoxylin was used as a nuclear counterstain. Images are at 40x magnification.
QRT-PCR primers detecting lineage specific markers were designed. *Col2a1* is specifically expressed by chondrocytes and is the major collagen in cartilage (Sandberg and Vuorio, 1987). *Col1a1* is expressed by cells undergoing cartilage and bone differentiation (Karsenty and Park, 1995, Suzuki et al., 2001). The transcription factor *Runx2* is expressed by terminally differentiating chondrocytes (Enomoto et al., 2000). *Osteopontin* is a structural protein expressed by terminally differentiated osteoblasts (Merry et al., 1993). QRT-PCR analysis showed that anole satellite cells in chondrogenic differentiation (blue bars) medium for 28 days showed significantly higher expression (p<0.05) of *Col1a1* (Figure 12 A), *Col2a1* (Figure 12 B) and *Runx2* (Figure 12 C) as compared to satellite cells in growth medium (black bars). Similarly treated mouse satellite cells showed no significant change in expression.

Since cartilage and bone differentiation share the same inducing factors (Wan and Cao, 2005), anole satellite cells were also cultured in osteogenic media. Culture of these cells in osteogenic (red bars) medium resulted in significantly (p<0.05) higher levels of expression of *Col1a1* (Figure 12 D) at 14 days. *Osteopontin* expression is detected at 7 days and maintained at 14 days in differentiation medium (Figure 12 E). Mouse satellite cells did not express these genes at a detectable level (data not shown). These data suggest that anole satellite cells can at least partially differentiate into non-muscle lineages.
Expression of bone and cartilage genes by anole satellite cells suggests that these cells might be able to autonomously produce factors that induce osteo/chondrogenesis. Therefore, anole satellite cells were assayed for expression of genes that induce cartilage and bone differentiation, BMPs, to determine whether their increased plasticity resulted from changes in expression of these important regulators of osteogenesis and chondrogenesis. BMP4 was not detected in mouse or anole satellite cells for all treatment regimes (data not shown). However, anole satellite cells cultured with osteogenic and chondrogenic media showed significantly (p<0.05) higher expression of both BMP2 (Figure 12 F) and BMP7 (Figure 12 G) in anole satellite cells at all time points measured. Mouse satellite cells that were similarly cultured did not express either gene at detectable levels (data not shown). Thus, these data suggest that anole satellite cells demonstrate a novel capability, not seen in mouse satellite cells, to express bone and cartilage specific markers suggesting that they are more plastic and might contribute to the regenerative capacity of A. carolinensis.
Figure 12: Anole satellite cells express chondrogenesis and osteogenesis marker genes
RNA was harvested from proliferating satellite cells (black bars) or cells treated with osteogenic (red bars) or chondrogenic (blue bars) differentiation media using TRIzol from 3 different plates. Varying time points were also collected, as indicated on the y-axis. cDNA was synthesized using SuperscriptIII (Invitrogen). Using gene specific primers that span an intron, QRT-PCR was done using Sybrgreen. Relative gene expression was calculated using ΔΔCt using proliferating satellite cells as the comparison control. All Ct values were Gapdh normalized. ** indicate samples that were significantly (p<0.05) different from control as determined by ANOVA with Tukey pairwise comparison test to confirm significance.
Anole satellite cells form calcified nodules in culture

Since anole satellite cells in culture express the marker *Osteopontin*, we hypothesized that anole satellite cells might lay down calcium deposits or nodules in culture. Anole and mouse satellite cells were differentiated in osteogenic medium for 28 days. 1% Alizarin red solution binds calcium and stains it red allowing for detection of calcium nodules deposited by osteoblasts (Dawson, 1926). Staining with Alizarin red indicated the presence of nodules in bone differentiated anole satellite cells (Figure 13, panel B). These nodules are not observed in similarly treated mouse satellite cells (Figure 13, panel D). Anole and mouse satellite cells in growth media for 28 days did not spontaneously develop nodules in culture (Figure 13, panels A and C), indicating that these nodules are specific to differentiated anole satellite cells. These data further support the observation that anole satellite cells demonstrate plasticity in culture not seen in mouse satellite cells.
Figure 13: Anole satellite cells form calcified nodules in osteogenic differentiation medium

Satellite cells were plated in either growth media (left panels) or osteogenic differentiation media (right panels) for 28 days. Anole satellite cells (**A** and **B**) and mouse satellite cells (**C** and **D**) were fixed with methanol and stained with 1% Alizarin Red to visualize calcium deposits. Anole satellite cells in osteogenic differentiation media (panel **B**) form large nodules that are indicative of calcium deposits in culture. Images are at 100x magnification.
Discussion

The ability of lizards to autotomize and consequently regenerate their tails is a striking example of effective tissue repair. The green anole, *Anolis carolinensis* has emerged as a new model system to study regeneration because of its tail regeneration capacity and the development of molecular tools made possible by the sequenced genome. In this study, we examine the primary muscle specific stem cell population, satellite cells, isolated from *A. carolinensis*. These cells exhibit characteristic satellite cell morphology, express marker genes such as *Pax7, Myf5, MyoD* and *Desmin* (Figure 9 and Table 2) and differentiate to form myotubes in culture (Figure 11).

In order to investigate whether these satellite cells could serve as a source of cells for the regenerating tail, we examined the plasticity of these cells in culture. Previous work has shown that mouse and human satellite cells can be induced to differentiate into adipocytes, chondrocytes and osteocytes (Wada et al., 2002) (Hashimoto et al., 2008). However, these studies either used media containing high (>100ng/ml) concentrations of morphogens in culture or stably transfected these cells to express tissue specific transcription factors (Katagiri et al., 1994) (Steinert et al., 2008). Thus, in order to determine native anole satellite cell plasticity, these satellite cells were not exposed to morphogens and mouse satellite cells were comparably treated as a control. Culturing anole
satellite cells in differentiation media resulted in an induction of tissue specific gene expression that was not mirrored in mouse satellite cells (Figure 11), suggesting that anole satellite cells are capable of differentiating into non-muscle lineages. We observed that Col1a1 is expressed by anole satellite cells when treated with chondrogenic or osteogenic media and Col2a1, the chondrogenesis specific collagen, is expressed in these cells when in chondrogenic medium. Runx2, a key osteoblast differentiation marker (Ducy et al., 1997) and Osteopontin, a structural protein secreted by osteoblasts (Merry et al., 1993) was significantly up-regulated by anole satellite cells when in differentiation medium as well. A. carolinensis satellite cells also form nodules in culture that stain positive for Alizarin red, which binds calcium (Figure 13). These data are a novel demonstration of anole satellite cell plasticity in culture.

Currently, a large amount of work is being carried out characterizing stem cells for human disease and injury intervention. However, true morphological regeneration in vivo of organs requires an understanding of the mechanisms controlling these processes. As musculoskeletal regeneration in human tissues is not observed, the study of other model systems that can regenerate is required to elucidate these mechanisms. Better understanding of the processes involved in regeneration will allow insights into how human stem cell research can be functionally implemented.
In conclusion, this study examines satellite cells isolated from *A. carolinensis*, a green anole that successfully regenerates its tail. *A. carolinensis* satellite cells demonstrated a previously uncharacterized capacity for increased plasticity in culture as compared to mouse. This work suggests that anole satellite cells are specified to a myogenic fate but not committed, unlike their mammalian counterparts. Thus, anole satellite cells might contribute to the regenerative capacity of *A. carolinensis*. Further work is required to elucidate the molecular mechanisms that allow increased plasticity of these satellite cells.

**Materials and Methods**

Satellite cell isolation and growth:

Satellite cells were isolated from adult *Anolis carolinensis* with minor modifications to protocol described in (Allen et al., 1997). Briefly, muscle was excised, trimmed of connective tissue, finely minced to a pulp, digested with 1.25mg of protease XIV (Sigma) for one hour at 37°C, triturated to extract satellite cells and passed through a 100µm nylon mesh. The cell suspension was spun down at 1500 x g for 10 mins and preplated in DMEM, 2% HS and 100µg/ml primocin (Invivogen) to remove fibroblasts and other debris for 2 hours. Purified satellite cells were grown on matrigel (BD Biosciences) in growth media, Hams F-10, 20% FBS, 10ng/ml bFGF (BD Biosciences) and 100µg/ml primocin.
Proliferation Assay:

For proliferation assays, anole and mouse satellite cells were plated in matrigel coated 96-well plates at $2 \times 10^4$ cells/well at day 0. Cells were trypsinized, stained with 0.4% trypan blue (CellGro) to exclude dead cells and counted using a hemocytometer every day for 6 days.

Differentiation Assay:

For muscle differentiation, satellite cells were allowed to reach >85% confluency and switched to differentiation media (DMEM, 2% HS, 100µg/ml primocin) every day for 3 days until large, fused myotubes were observed. Osteogenic and chondrogenic differentiation was carried out as described in (Lu et al., 2010) with modifications. For osteogenic differentiation, satellite cells were grown in Ham’s F-10, 15% FBS, 0.1 µM dexamethasone, 50µM ascorbate 2-phosphate, 10mM β-glycerophosphate and 100µg/ml primocin for 2 weeks. RNA was harvested at 7 and 14 days for analysis of gene expression by QPCR. For chondrogenic differentiation, satellite cells were grown in until highly dense and then switched into Ham’s F-10, 1% FBS, 6.25 mg/ml insulin, 10ng/ml TGFβ1, 50nM ascorbate-2-phosphate and 100µg/ml primocin.
Quantitative RT PCR:

Satellite cells were lysed in Trizol (Invitrogen) and RNA extracted as per manufacturer's protocol. RNA was treated with DNase I (New England Biolabs, Ipswich, MA) and complementary DNA (cDNA) was made from each RNA sample using SuperScript III Reverse Transcriptase (Invitrogen). cDNA was then quantified using transcript specific, intron spanning primers and quantitative PCR using SYBR Green (Eurogentech). Samples are normalized to Gapdh for comparative analysis using ΔΔCt method.

Immunofluorescence:

Cells were plated on a coverslip coated with matrigel and allowed to grow overnight in growth media at 30°C. Cells were fixed using cold methanol at -20°C for 8 minutes. Pax7 was detected using anti-Pax7 antibody (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) at 1:250 dilution and anti-mouse IgG FITC secondary antibody (Sigma).
CHAPTER 4

CONCLUSIONS

Adult muscle specific progenitor stem cells, satellite cells are required for postnatal muscle repair. In response to muscle damage, quiescent satellite cells become activated, undergo proliferation and then either return to a quiescent state or differentiate (Figure 1). Notch signaling has been shown to be an important part of this process and Numb, an inhibitor of Notch signaling is asymmetrically localized in satellite cells suggesting that this protein might play a role in the binary cell fate decision (Shinin et al., 2006). In these studies, the role of NUMB in satellite cell mediated muscle repair was studied using conditional Numb null mice. Numb deficient mice demonstrated improper muscle repair compared to control muscle when injected with cardiotoxin, as characterized by collagen deposition, increased endomysial space and smaller myofibers (Figure 3). Since active Notch signaling is most often associated with proliferation, loss of the inhibitor Numb would be predicted to cause an increase in progenitor cell numbers. In order to examine the role of NUMB in satellite cell function, satellite cells were isolated from conditional Numb null animals and Numb was successfully excised in culture (Figure 4). Contrary to the expected result, Numb deficient satellite cells have a proliferation defect (Figure 6). Mstn, the muscle growth inhibitor, was highly up-regulated in these cells (Table 1). Knockdown of Mstn using transcript specific RNAi rescued this phenotype (Figure 8), suggesting that
Mstn up-regulation inhibits proliferation in these cells. This work suggests a novel role for NUMB in satellite cell function mediated by an interaction with the important muscle growth inhibitor, MSTN.

How a lack of Numb leads to up-regulation of Mstn is unknown. SMAD3 is the effector transcription factor downstream of MSTN signaling. Interestingly, Smad3−/− mice show increased Mstn expression (Ge et al., 2011) and active Notch signaling blocks SMAD3-dependent induction of cell cycle inhibitors such as p21 (Carlson et al., 2008). This suggests cross talk between Notch signaling components and MSTN. Future work will examine if phosphorylated SMAD3 is a target of proteosomal degradation by NUMB by examining mRNA and protein levels of this protein. Numb-deficient satellite cells form myotubes but it is clear that a loss of NUMB results in aberrant myotubes as characterized by their reduced size and decreased MHC expression (Figure 5). Previous work in progenitor neuronal lineages has shown that NUMB65 and NUMB66 promote differentiation at the expense of proliferation and the inverse is true for NUMB71 and NUMB72 (Verdi et al., 1999). Loss of NUMB might cause improper differentiation resulting in hypotrophic myotubes (Figure 5, panel D). By transfecting individual Numb isoforms into differentiating satellite cells, we can examine if this defect is rescued by NUMB and the specific isoforms that might be important in this process.
Satellite cells contribute to muscle healing in mammals but can not regenerate muscle. Reptiles are evolutionarily the closest animals to mammals that retain regenerative capacity; they are able to regenerate tails after autotomy. The regenerated reptile tail is functional and contains muscle, fat, cartilage, nerves and skin. Mammals can regenerate skin to some extent but de novo replacement of muscle, cartilage and nerves is not observed. These studies described, for the first time, successful isolation and culture of satellite cells from the reptile *Anolis carolinensis*. Satellite cells isolated from these animals are shown to be comparable to mouse satellite cells in their growth rates (Figure 10), gene expression profiles (Table 2) and in their capacity to form myotubes in culture (Figure 11). Since satellite cells constitute one possible source of cells for the regenerating tail, we examined if these cells could contribute to non-muscle tissues found in the regenerated anole tail. Satellite cells from mammals have been driven into non-muscle lineages, by administration of large doses of morphogens in culture. In order to evaluate if anole satellite cells could contribute to the regenerating anole tail, we did not treat our cells with transforming proteins. Interestingly, *A. carolinensis* satellite cells showed increased plasticity in culture without morphogen treatment (Figure 12). Anole satellite cells expressed osteogenic and chondrogenic markers such as *Runx2*, *Col1a1*, *Col2a1*, *Osteopontin* and *BMP2*. Similarly treated mouse satellite cells do not show expression of these markers. These data are a novel observation of plasticity of satellite cells
and suggest that satellite cells contribute to the regenerative capacities of the lizard.

Further work is required to determine if in vivo, satellite cells contribute to the regenerating tail. Immunological staining for markers of satellite cells (such as PAX7) in the regenerating anole tail will indicate if satellite cells contribute to muscle or non-muscle tissues in the regenerating tail. However, since PAX7 is an early marker for satellite cells, it might not be detected as satellite cells differentiate. Alternatively, radio or dye labeled satellite cells could be injected into anoles prior to autotomy. Regenerating tails can be examined to determine if satellite cell specific labeling can be found in non-muscle tissues of the newly formed tail. Increased plasticity of anole satellite cells might be due to changes in gene expression. Analysis of anole and mouse satellite cell transcriptomes with RNA-Seq will help identify differential gene expression between these cell types. After confirmation of differences in gene expression using QRT-PCR, expression of these candidate genes can be knocked down in culture using interference RNA. Changes in plasticity of RNAi treated anole satellite cells will be used to determine if these genes contribute to anole regeneration capacity. Increased plasticity might also be locally controlled by regulators of cartilage and bone differentiation. Analysis of changes in regulatory elements in promoter regions of inducers of bone and cartilage differentiation might also explain increased anole satellite cell plasticity.
REFERENCES


De Strooper, B., Annaert, W., Cupers, P., Saftig, P., Craessaerts, K.,


(BMP) receptor oligomerization determines different BMP-2 signaling pathways. J. Biol. Chem. 277, 5330–5338.


Vasyutina, E., Lenhard, D. C., Wende, H., Erdmann, B., Epstein, J. A.,


Zammit, P. S. (2008). All muscle satellite cells are equal, but are some more equal than others? Journal of Cell Science 121, 2975–2982.


