IMPACTS OF BMP6 ON MYOGENIC CELL PROLIFERATION, DIFFERENTIATION, AND SATELLITE CELL POPULATION

By

WENLI SUN

A THESIS PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE UNIVERSITY OF FLORIDA

2010
ACKNOWLEDGMENTS

It is an honor to thank those who made this thesis possible. First of all, I owe my deepest gratitude to the chair of my committee, Dr. Sally Johnson, who gave me the chance to study at the University of Florida and work under her direction. This thesis would not have been possible without her guidance and support. I also thank Dr. Alan Ealy and Dr. Peter Hansen, for serving on my supervisory committee and for making excellent suggestions and ideas.

It is also a pleasure for me to show my gratitude to my former and present colleagues: Ju Li, Dillon Walker, Diana Delgado, John Michael Ganzalez, Sarah Reed, and Marni Lapin. Thank you for kindly providing me the necessary help for my work.

I would like to thank my beloved families and friends for being my back-up all the time. The love from my parents was always a source of courage. It is my friends that keep me smiling through the hard times. Special thanks to Hsiu and Shadow the cat; thank you for your company which comfort me a lot.

Lastly, I offer my regards and blessings to all of those who supported me in any way during the completion of my thesis.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>3</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>5</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>6</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>1 LITERATURE REVIEW</td>
<td>7</td>
</tr>
<tr>
<td>Satellite Cells: Definition and Functions</td>
<td>7</td>
</tr>
<tr>
<td>Extracellular Surface Marker-Associated Satellite Cell Identification</td>
<td>8</td>
</tr>
<tr>
<td>Molecular Identification of Satellite Cells</td>
<td>10</td>
</tr>
<tr>
<td>Satellite Cell Self-Renewal and Progenitor Production</td>
<td>12</td>
</tr>
<tr>
<td>Microenvironmental Control of Satellite Cell Biology</td>
<td>14</td>
</tr>
<tr>
<td>Notch, Wnt and Self-Renewal</td>
<td>15</td>
</tr>
<tr>
<td>Hepatocyte Growth Factor and Activation</td>
<td>17</td>
</tr>
<tr>
<td>Growth Differentiation Factor 8</td>
<td>20</td>
</tr>
<tr>
<td>Bone Morphogenetic Protein 6</td>
<td>22</td>
</tr>
<tr>
<td>2 MATERIALS AND METHODS</td>
<td>25</td>
</tr>
<tr>
<td>Bovine Satellite Cell Isolation</td>
<td>25</td>
</tr>
<tr>
<td>Cell Culture</td>
<td>25</td>
</tr>
<tr>
<td>Immunocytochemistry</td>
<td>26</td>
</tr>
<tr>
<td>Alkaline Phosphatase Histology</td>
<td>27</td>
</tr>
<tr>
<td>Western Blots</td>
<td>27</td>
</tr>
<tr>
<td>Luciferase Reporter Assay</td>
<td>28</td>
</tr>
<tr>
<td>BMP6 RT-PCR</td>
<td>29</td>
</tr>
<tr>
<td>Statistics</td>
<td>29</td>
</tr>
<tr>
<td>3 RESULTS</td>
<td>30</td>
</tr>
<tr>
<td>BMP6 is Expressed in Bovine Skeletal Muscle</td>
<td>30</td>
</tr>
<tr>
<td>BMP6 Affects Distinct Aspects of Satellite Cell Myogenesis</td>
<td>30</td>
</tr>
<tr>
<td>Repression of BSC Myogenesis is Independent of Transdifferentiation</td>
<td>32</td>
</tr>
<tr>
<td>4 DISCUSSION</td>
<td>46</td>
</tr>
<tr>
<td>LIST OF REFERENCES</td>
<td>51</td>
</tr>
<tr>
<td>BIOGRAPHICAL SKETCH</td>
<td>62</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-1</td>
<td>Bovine muscle tissue expresses BMP6</td>
<td>34</td>
</tr>
<tr>
<td>3-2</td>
<td>BMP6 phylogenetic tree</td>
<td>35</td>
</tr>
<tr>
<td>3-3</td>
<td>BMP6 inhibits EdU incorporation</td>
<td>36</td>
</tr>
<tr>
<td>3-4</td>
<td>BMP6 changes BSC subpopulation proportions</td>
<td>37</td>
</tr>
<tr>
<td>3-5</td>
<td>BMP6 inhibits myofiber formation</td>
<td>38</td>
</tr>
<tr>
<td>3-6</td>
<td>BMP6 inhibits differentiation of BSC and myoblasts</td>
<td>40</td>
</tr>
<tr>
<td>3-7</td>
<td>BMP6 induces alkaline phosphatase (ALP) activity in C2C12 myoblasts but not 23A2 myoblasts and BSC</td>
<td>41</td>
</tr>
<tr>
<td>3-8</td>
<td>BMP6 activates SMAD1/5/8 in BSC, 23A2 and C2C12 myoblasts</td>
<td>43</td>
</tr>
<tr>
<td>3-9</td>
<td>BMP6 stimulates transcription of BRE-Luc</td>
<td>45</td>
</tr>
<tr>
<td>4-1</td>
<td>Illustration of satellite cell subpopulations and myogenesis</td>
<td>50</td>
</tr>
</tbody>
</table>
Bone morphogenetic protein 6 (BMP6), a member of TGF-beta superfamily, plays an important role in modulating epithelial and neural tissue development. The expression of BMP6 in young (<7 days) bovine semimembranous muscle tissue suggests a potential regulatory effect in bovine muscle. Exogenous BMP6 treatment was examined in three different myogenic cell cultures: bovine satellite cells, 23A2 mouse myoblasts, and C2C12 mouse satellite cells. The treatment effect was confirmed by BRE-Luc activity in all cells. BMP6 caused different responses in different cells. The proliferation rate was decreased by BMP6 in BSC, 23A2 and C2C12 cells. Myogenic differentiation and fiber formation were suppressed in BSC, 23A2 and C2C12 cells. Alkaline phosphatase activity was induced by BMP6 in C2C12 but not in 23A2 or BSC. Furthermore, BMP6 treatment changed proportion of cells defined by Pax7 and Myf5 in BSC. In all 3 types of cells, western blotting was used to demonstrate phosphorylation and activation of SMAD 1/5/8. These data indicate that BMP6 signals through SMAD1/5/8 to regulate myogenic cell proliferation and differentiation.
Satellite Cells: Definition and Functions

In 1961, Alexander Mauro described a minor population of heterochromatin dense cells positioned immediately adjacent to the mature muscle fiber in frogs (Mauro, 1961). These cells, which he termed satellite cells, reside immediately beneath the fiber basal lamina and appear quiescent. He proposed that satellite cells were dormant embryonic myoblasts or unknown infiltrating cells that may explain “the vexing problem of skeletal muscle regeneration”. While numerous groups had noted the unique ability of muscle to repair itself, the source of new muscle fibers and myonuclei within the fibers remained unresolved.

Bintliff and Walker (1960) reported that neofibers formed during mouse skeletal muscle regeneration did not contain $^3$H-thymidine labeled nuclei when the isotope was administered 2-3 days after damage, leading the group to conclude that myonuclei are mitotically inactive (Bintliff and Walker, 1960). These results were extended to chick embryonic myofiber formation by Stockdale and Holtzer (1961) who found that somite myofibers fail to incorporate radiolabeled thymidine (Stockdale and Holtzer, 1961). However, the source of the proliferative cells that allow postnatal muscle growth and repair remained unclear.

In 1970, Moss and LeBlond performed a time course study of $^3$H-thymidine incorporation into muscle nuclei of rapidly growing rat pups (Moss and Leblond, 1970). Conclusive evidence was obtained by electron microscopy demonstrating that satellite cells were mitotically active and capable of fusion with adjacent muscle fibers. Thus, Mauro’s contention that satellite cells are a source of myonuclei was validated.
The ability of satellite cells to reside within the muscle throughout the lifespan of an individual enables both growth and repair capabilities. The numbers of satellite cells in rat muscle decline from birth to adulthood coincident with increased myonuclei and muscle mass (Cardasis and Cooper, 1975). However, their supply is never abrogated with advanced age. Electron microscopy revealed that a small percentage of satellite cells, less than 1% of total muscle nuclei, are retained in the muscles of people over the age of 70 (Schmalbruch and Hellhammer, 1976). These pioneering efforts provided the first glimpse of what is now considered the adult muscle stem cell.

**Extracellular Surface Marker-Associated Satellite Cell Identification**

Satellite cells, historically, are identified by their physical position under the basal lamina adjacent to the muscle fiber. Isolation and culture of the population often was hindered by the presence of non-fusing cells that most investigators classified as fibroblasts. Early attempts at molecular definition of rodent satellite cells revealed that many of these cells expressed the structural protein, desmin. Satellite cells isolated from juvenile rat pups express desmin prior to induction of the myogenic gene regulatory network and differentiation (Foster et al., 1987; Kaufman and Foster, 1988). These results further substantiated that satellite cells are myogenic precursor cells that are distinct from embryonic myoblasts. Similar to the rat, proliferating cultures of human satellite cells express the intermediate filament protein (van der Ven et al., 1992). Although greater than 95% of human satellite cells contain desmin, progeny of these cells exhibit divergent myogenic potential (Baroffio et al., 1995).

The majority of human satellite cells commit to differentiate as indicated by expression of skeletal actin, myosin and fusion. By contrast, a small number of clonal satellite cells retain desmin expression but fail to proceed into the terminal differentiation
program. The non-fusing desmin expressing population is the first report of a muscle-derived stem cell. This cell is retained as a mononucleate in the presence of differentiation permissive conditions (Baroffio et al., 1996). Subculture of these cells leads to the emergence of myoblast progeny capable of biochemical and morphological differentiation as well as self-renewal.

The isolation and characterization of desmin-positive myogenic precursors from humans and rodents provided the initial evidence for heterogeneity within the satellite cell compartment. Indeed, four desmin-expressing satellite cell subpopulations are found in humans with each demonstrating variable degrees of differentiation capabilities (Edom-Vovard et al., 1999). However, desmin is not a cross-species marker of satellite cells. Primary cultures of bovine satellite cells are less than 15% desmin immunopositive (Allen et al., 1991). Importantly, these cells fail to incorporate thymidine analogs suggesting they are at an early stage of terminal differentiation.

The inability of desmin to denote all satellite cells and the documented level of heterogeneity within the compartment underscored the search for universal markers of the adult muscle population. Cell surface proteins, including integrins, adhesion molecules and extracellular matrix glycoproteins, were explored for their value as isolation tools for satellite cell enrichment. Postnatal rat muscle fibers exhibit limited surface expression of α7-integrin but a substantial number of putative satellite cells are immunoreactive for the adhesion molecule (Song et al., 1992). It was further noted that antibodies against a splice variant of α7-integrin are effective satellite cell enrichment tools (Ziober et al., 1993). Human myoblasts obtained by fluorescence activated cell sorting (FACS) for α7-integrin are 95% myogenic in nature (Blanco-Bose et al., 2001).
In addition to serving as an isolation aid, the laminin receptor formed by α7β1-integrin association plays a critical role in migration of myogenic cells in vitro (Schober et al., 2000; Yao et al., 1996). While α7β1-integrin serves as a convenient marker of satellite cells and myoblasts, it is not exclusive to myogenic cells and enrichment for muscle precursors using anti-α7β1 integrin is species-specific (Flintoff-Dye et al., 2005; Gardiner et al., 2005; Kallestad and McLoon, ; Mayer et al., 1997; Welser et al., 2007a; Welser et al., 2007b).

A host of extracellular matrix-associated molecules used for satellite cell purification has evolved substantially since the early efforts employing anti-α7-integrin. Immunohistochemical elucidation of muscle progenitors in postnatal animals often employs antibodies directed against neural cell adhesion molecule (NCAM) (Cashman et al., 1987), M-cadherin (Irintchev et al., 1997), c-Met (Tatsumi et al., 1998) and nestin (Day et al., 2007). Each antibody denotes mononucleated cells beneath the fiber basal lamina that become mitotically active during muscle regeneration. Isolation of satellite cells for therapeutic intervention of muscle damage and disease typically utilizes Hoechst dye exclusion and/or immunofluorescent enrichment with flow cytometry. FACS methodology includes combinations of antibodies for syndecan-3 and -4 (Cornelison et al., 2001; Cornelison et al., 2004), surface heparin sulfate proteoglycans, CXCR4 (Sherwood et al., 2004), a chemokine receptor, SM/C2.6 (Fukada et al., 2004), a poorly characterized surface antigen, and CD34 (Montarras et al., 2005), a sialomucin protein that denotes progenitor cells.

**Molecular Identification of Satellite Cells**

Substantive advances in the field of satellite cell biology occurred following elucidation of key transcription factors involved in lineage commitment of muscle
precursor. Early research determined that the adult satellite cell expressed coordinately members of the myogenic regulatory factor (MRF) family (Smith et al., 1994). The MRFs, Myf5, MyoD, myogenin and MRF4, exhibit the unique ability to initiate the skeletal muscle gene expression program in non-muscle cells in vitro (Chanoine et al., 2004). Myf5 and MyoD are expressed during early mouse embryogenesis and are responsible for establishment of the myogenic lineage. Mice genetically ablated of these two transcription factors die in utero and are devoid of a myoblast population (Rudnicki et al., 1993).

As the skeletal myoblast transits into the myogenic program, it begins to express myogenin, the requisite transcriptional mediator for terminal differentiation. Myogenin-/- mice die shortly after birth; they contain myoblasts but are deficit in contractile-competent muscle fibers (Hasty et al., 1993). With regards to satellite cells, Myf5 and MyoD are expressed during the early proliferative period in vitro followed by down-regulation of the genes and up-regulation of myogenin. Due to the identical expression pattern found in embryonic myoblasts, it was thought that the satellite cell may represent an arrested embryonic myoblast. However, satellite cells are retained throughout the lifetime of an individual due to self-renewal of the population, a feature distinct from the embryonic myoblast.

To identify genetic factors critical to satellite cell development and postnatal function, Seale et al. (2000) performed representational difference analysis (RDA) to enrich for transcripts unique to satellite cells. The genetic screen identified Pax7 as an abundant transcript in the adult muscle precursor population. Pax7 is a paired box transcription factor orthologous to Pax3, a regulatory factor expressed prior to the MRFs
and required for initiation of Myf5 transcription (Maroto et al., 1997). Mice null for Pax7 typically die prior to weaning with both neural and muscle defects (Mansouri et al., 1996; Seale et al., 2000). Electron microscopy revealed that the Pax7-/- skeletal muscle is severely compromised in satellite cell numbers. However, a portion of the genetic null mice survive to adult with no apparent reduction in muscle fiber numbers or cross-sectional area (Oustanina et al., 2004). Injection of cardiotoxin, a myonecrotic agent, into Pax7-/- hindlimb muscles caused a severe reduction in neofiber formation and muscle regeneration. Serial culture of putative satellite cells isolated from Pax7-/- revealed a decline in the numbers of muscle progenitor cells and their ability to form fibers when compared to heterozygous controls. Thus, it was concluded that Pax7 is not required for specification of the lineage but is necessary for self-renewal of satellite cells.

An elegant confirmation and extension of these findings was performed using conditional ablation of the transcription factor. Genetic ablation of Pax7-/- in young mice (P60-90) did not disrupt growth, regeneration or satellite cell self-renewal (Lepper et al., 2009). By contrast, conditional removal of Pax7 in utero or during the early juvenile period (<P10) resulted in compromised satellite cell numbers and repair function analogous to germline null mice. Two important findings are noted. First, Pax7 is required through initial entry of satellite cells into G0. Secondly, adult muscle satellite cells acquire a regulatory network that is not dependent upon Pax7 for either self-renewal or myogenicity.

**Satellite Cell Self-Renewal and Progenitor Production**

Due to the sustained regenerative capacity of skeletal muscle tissue over the lifetime of an individual, the muscle stem cell(s) present must both self-renew and
generate a pool of progenitors. Early work in both rodent and human models indicated that not all satellite cells were functionally equivalent, suggestive of distinct stem and progenitor subgroups. Mixed populations of satellite cells with different cell cycle kinetics were reported in rats (Schultz, 1996). Also, primary cultures of human satellite cells display different proliferation and differentiation capabilities (Baroffio et al., 1995).

In the search for protein fingerprints unique to quiescent muscle progenitors, Beauchamp (2000) noted a degree of heterogeneity within the satellite cell pool based upon Myf5 expression. Myofibers with attached satellite cells isolated from Myf5-nLacZ mice revealed that two subpopulations exist based upon differential expression of the transgene. All satellite cells expressed CD34 and M-cadherin but a minor myogenic population did not exhibit β-galactosidase activity, the assay product for nLacZ. Kuang et al (2007) extended these observations to the Pax7-expressing satellite cells with 90% of the population exhibiting expression of Myf5 and 10% expressing Pax7-only. Moreover, Pax7-only muscle cells divide asymmetrically, a hallmark of stem cell self-renewal, to yield a daughter cell expressing both Pax7 and Myf5. Transplantation of Pax7-only cells into the tibialis anterior of Pax7-/- mice revealed that the cell could both restore muscle growth and re-populate the niche. By contrast, Pax7+/Myf5+ expressing satellite cells were unable to reconstitute the sublaminar pool and exhibited only limited amounts of muscle repair. These efforts provide a framework for definition of satellite cell stem and progenitors based upon differential Myf5 expression.

Although Pax7 is regarded as the definitive marker of adult muscle satellite cells, it is not exclusive. A portion of satellite cells exhibit Pax3 expression and others are defined by Pax3 and Pax7 co-expression (Otto et al., 2006; Relaix et al., 2005). Pax3
and Pax7 exhibit overlapping, as well as unique, expression patterns during embryogenesis in rodents and chicks (Borycki et al., 1999; Goulding and Paquette, 1994; Williams and Ordahl, 1994). Moreover, the two control distinct elements within embryonic and adult myogenesis. Pax3 initiates transcription of Myf5 to establish the early myogenic lineage during embryogenesis, an event that precedes MyoD expression (Tajbakhsh et al., 1997). Mice homozygous null for both Pax3 and Myf5 lack body muscles and MyoD expression. Sequential activation of Myf5 and MyoD does not occur in all satellite cells indicating at least two distinct subpopulations exist (Cooper et al., 1999).

Following cardiotoxin-induced injury in mice, satellite cells express Myf5, MyoD or a combination of the two MRFs. Unlike Cornelison and Wold (1997), Myf5 was not detected in quiescent satellite cells; the protein was evident only upon activation. The ability of Myf5 to denote quiescent satellite cells was explored further using heterozygous Myf5-nLacZ mice, which contain nuclear LacZ knocked into the one allele of Myf5 (Beauchamp et al., 2000). β-galactosidase expressing M-cadherin immunopositive satellite cells were evident in non-injured adult muscles supporting the hypothesis that Myf5 is a marker of quiescent muscle progenitors in vivo. Satellite cells isolated from heterozygous Myf5-nLacZ mice and expanded in vitro successfully engraft into diseased muscle (mdxnu/nu) with a small number assuming the satellite position (Heslop et al., 2001).

**Microenvironmental Control of Satellite Cell Biology**

Numerous growth factors, morphogens and hormones exert effects on both muscle fibers and satellite cells. Several members of the fibroblast growth factor (FGF) superfamily serve as potent mitogens while suppressing myofiber formation.
Platelet-derived growth factor (PDGF) exerts effects similar to the FGFs and may be one of the first blood-borne growth factors delivered to sites of myotrauma (Christov et al., 2007). Insulin-like-growth factor I (IGF-I) has little effect on satellite cell proliferation but strongly supports myoblast fusion into mature fibers (Clemmons, 2009). Although these growth factors are important to satellite cell actions and muscle function, they are often delivered systemically and are not regarded as niche factors for the purposes of this discussion.

**Notch, Wnt and Self-Renewal**

The niche localized signals that direct self-renewal and progenitor development remain poorly understood. Asymmetric cell division leading to fate decisions occurs in many organisms and tissues and commonly employs a Notch signal. Notch, a transmembrane receptor, binds Delta and Jagged ligands leading to γ-secretase cleavage of the intracellular domain (ICD) (Fortini, 2009). ICD proteolytic maturation allows for nuclear translocation and modification of gene transcription. The Notch pathway is intact in mouse satellite cell: myofiber explant cultures and receptor activation causes increased proliferation (Conboy and Rando, 2002). Immunostaining for the Notch inhibitor, Numb, demonstrated a portion of the dividing satellite cells exhibited asymmetric distribution of the protein. Importantly, the daughter cell with intense Numb localization failed to contain detectable Pax3 indicating a more committed progenitor cell. No differential Numb localization was observed in Pax7-expressing satellite cells. Pax7+/Myf5- satellite cells expressed abundant amounts of Notch-3 by comparison to Pax7+/Myf5+ progenitors, which express greater amounts of Delta-1 transcripts (Kuang et al., 2007). In vivo BrdU pulse labeling experiments followed by myofiber explant culture demonstrated that asymmetric division of attached satellite
cells involved co-segregation of template DNA and Numb to the putative muscle stem cell (Shinin et al., 2006).

The importance of Notch inhibition via Numb as a determinant of progenitor commitment was challenged by experiments with targeted mis-expression in mouse embryos. Ectopic expression of Numb in Pax3 and Pax7 somitic cells prior to progenitor fate commitment revealed that Numb increased the numbers of Pax3+/Pax7+ stem cells, contrary to expectations (Jory et al., 2009). While it is safe to state that Notch signals affect myogenic decisions, it remains unclear if the various satellite cell subpopulations respond to the fate determinant analogously.

The Drosophila Wingless gene and the vertebrate homolog, Int-1, are commonly referred to as Wnts. In mammals, this large family of secreted proteins binds to frizzled (Fz) receptors to elicit canonical responses through nuclear β-catenin accumulation as well as non-canonical effects that include activation of Rac and Rho GTPases (Sethi and Vidal-Puig). Early work using chick somite explant cultures detailed the ability of Wnt1, produced by the neural tube, to activate Myf5 in the dorsal aspects of somite committing cells to the myogenic lineage (Munsterberg and Lassar, 1995; Stern, 1995). A similar fate decision occurs through surface ectoderm-derived Wnt7a induction of MyoD in the dermamyotome compartment of the somite (Tajbakhsh et al., 1998). These early fate decisions were extrapolated to regenerating muscle and satellite cells. Wnt5a, 5b and 7a are transcribed by primary mouse myofiber explant cultures and treatment of CD45+/Sca1+ hematopoietic progenitors with a cocktail of the Wnts is sufficient to instill the myogenic gene network (Polesskaya et al., 2003). The authors conclude that niche
production of the Wnts during muscle regeneration serves to recruit non-myogenic cells into the lineage and improve regenerative capabilities.

Direct involvement of Wnts on satellite cell biology was reported by Steelman et al (2006) who found that Wnt4 acts a mitogen for mouse satellite cells en masse. By contrast, Otto et al. (2008), using single fiber explant cultures, found that Wnt4 inhibits proliferation of the associated satellite cells while Wnt1, 3 and 5a increased satellite cell proliferation (Otto et al., 2008).

The ability of the Wnts to alter satellite cell myogenesis appears to be age-dependent. Satellite cells from old mice tend to lose their myogenicity at the expense of a fibroblast-like lineage (Brack et al., 2007). In contrast to embryonic myoblasts, Wnt signaling causes transdifferentiation of aged satellite cells. The identity of the circulating Wnt or niche-localized Wnts responsible for the fate modification remain unknown. Interestingly, the fate altering Wnt activity is absent from the serum of young mice.

**Hepatocyte Growth Factor and Activation**

It was noted that satellite cells attached to intact, viable muscle fibers exited quiescence sooner if the culture contained damaged or dead fibers (Bischoff, 1986). Crude preparations of crushed muscle extract (CME) contained a mitogen that shortened the time to G1/S phase in satellite cells cultured in vitro and increased the numbers of proliferative satellite cells following injection in vivo. The unknown activator and mitogen elicited similar activity on rat satellite cells as hepatocyte growth factor (HGF) (Allen et al., 1995). HGF reduced the time delay between G0 and G1/S in cultures of satellite cells isolated from mature rats, analogous to CME. Due to the functional similarities and satellite cellexpression of c-Met, the HGF receptor, Allen et al. (1995) postulated that HGF was the activator of CME. Validation of HGF as the satellite
cell activation factor was provided by Tatsumi et al (1998) whereby it was demonstrated that HGF was present in CME and that immunodepletion of HGF from CME prevented satellite cell activation.

HGF is synthesized and released by the satellite cell to create an autocrine loop that facilitates proliferation (Sheehan et al., 2000) and prevents precocious differentiation (Gal-Levi et al., 1998). Due to its inhibitory effects on fiber formation, the therapeutic potential of HGF may be limited. Injection of the growth factor into regenerating skeletal muscle caused an increase in the numbers of proliferating satellite cells but also suppressed neofiber formation (Miller et al., 2000). The inhibitory actions of HGF toward myoblast fusion and differentiation are mediated, in part, through c-Met initiated intracellular signals that culminate in up-regulation of Twist, a basic helix-loop-helix transcriptional repressor (Leshem et al., 2000). Translational inhibition of Twist mRNA with a putative antisense RNA molecule partially restores biochemical and morphological parameters of myogenesis to chick satellite cells treated with HGF.

The importance of HGF to satellite cell function during myotrauma and regeneration often overshadows the influence of the regulatory protein as a mediator of muscle hypertrophy. Resistance exercise, stretch and the normal process of muscle growth are dependent upon the activation of satellite cells. Rat satellite cells that received a 2-hr mechanical stretch stimulus re-entered the cell cycle sooner than non-stretched cells and the improved activation kinetics were prevented by immunosorption of the autocrine HGF (Tatsumi et al., 2002; Tatsumi et al., 2001). Young men that performed an acute bout of eccentric exercise to achieve contraction-induced muscle
damage released more active HGF from the muscle tissue (O'Reilly et al., 2008). In turn, the active HGF stimulated satellite cell activation and proliferation.

The release of HGF from the muscle fiber and receptor docking on the adjacent satellite cell rapidly alters cell cycle dynamics. Expeditious release of HGF from the extracellular matrix reservoir necessitates both a shear detection mechanism and a proteolytic system for processing and activation of the growth factor.

Anderson (2000) proposed that nitric oxide (NO), an abundant, diffusible molecule found in muscle, participated in the initial signal for HGF-initiated activation of satellite cells. Treatment of dystrophic mice (mdx) with L-NAME, a chemical inhibitor of the NOS enzyme response for NO production, resulted in a substantial delay in muscle regeneration that was attributed to a block in HGF release from the fiber ECM and a subsequent blunting of satellite cell activation (Anderson, 2000). These results were extended by demonstration that stretch-induced HGF release from satellite cells is prevented by inhibition of NO production thereby, delaying in vitro activation. Although release of HGF is instrumental to receptor mediated actions on the satellite cell, these experiments did not address the proteolytic processing of HGF or its shedding from the ECM. HGF is synthesized as a large precursor protein that requires proteolytic processing into α and β chains that assemble into the functional heterodimeric HGF (Naka et al., 1992). Tatsumi and Allen (2004) demonstrated that HGF is tethered to the myofiber ECM in both an active and inactive configuration. Pro-HGF is rapidly cleaved to its active form upon incubation with crushed muscle extract indicating that a matrix-associated protease system is present for production of mature HGF. Importantly,
treatment of intact muscle with nitroprusside, a NO donor, resulted in the release of mature HGF heteromeric complexes from the matrix (Tatsumi and Allen, 2004).

HGF release likely is mediated through NO activation of matrix metalloproteinases (MMPs), a family of endopeptidases that degrade multiple ECM components. Treatment of satellite cells with TIMP, an inhibitor of MMPs, prevented HGF release from the ECM during cyclic stretch and blunted activation (Yamada et al., 2008). These results provide the basis for a working model of satellite cell activation in health and disease that includes near instantaneous synthesis and release of NO from the myofiber leading to MMP-mediated release of HGF, the growth factor required for exit from G0.

**Growth Differentiation Factor 8**

One of the best examples of unrestricted skeletal muscle size is the Belgian Blue breed of cattle. These animals are noted for their massive amounts of muscle deposition to the extent that they are often referred to as “double-muscled”. However, the animals do not possess duplicate muscles but simply contain twice as many muscle fibers per muscle (Ashmore et al., 1974; Swatland, 1974; Swatland and Kieffer, 1974). Due to the extreme amounts of muscle, problems with dystocia and unconventional carcass parameters, this breed has had limited acceptance as a big cattle breed in the United States.

In 1997, a new member of the transforming growth factor beta (TGF-β) superfamily, referred to as growth and differentiation factor 8 (GDF8) or myostatin, was described (McPherron et al., 1997). The gene is highly expressed in skeletal muscle during embryogenesis through adulthood. Mice genetically null for GDF8 exhibited 2-3 times the amount of muscle found in wildtype animals. The phenotypic resemblance of the GDF-/- mice to double muscled cattle provided a causative explanation for the
excess muscle hypertrophy. Sequence analysis of GDF8 in Belgian Blue cattle demonstrated an 11-bp deletion in the coding sequence for the bioactive carboxy-terminus leaving the protein inactive (McPherron and Lee, 1997). Pietmontese, another double-muscled breed, contain a point mutation in GDF8 that leads to production of a biologically inactive protein. From these results it was concluded that GDF8 is a negative regulator of muscle size.

GDF8 is synthesized as a propeptide that requires cleavage of the amino-terminal domain for bioactivity (Thies et al., 2001). The prodomain acts to inhibit GDF8 and injection or ectopic expression of the peptide enhances muscle size and attenuates the severity of muscle disease and cachexia (Bogdanovich et al., 2002; Whittemore et al., 2003; Zimmers et al., 2002). Although GDF8 circulates systemically, its major actions are regarded as autocrine and paracrine. GDF8 exerts its effects through the activin receptor IIb (ActRIIb) and includes phosphorylation and activation of Smad2 and Smad3 (Rebbapragada et al., 2003; Walsh and Celeste, 2005). Mice expressing a dominant negative form of ActRIIb in skeletal muscle display a 4-fold increase in muscle mass by comparison to the 2-fold increase observed in GDF8-/- animals (Lee and McPherron, 2001). The increased severity suggests that additional ActRIIb ligands participate in the regulation of muscle fiber size. GDF11, a structurally similar subfamily member to GDF8, does not modulate muscle size in the embryonic or neonatal mouse (McPherron et al., 2009; McPherron et al., 1999). Mice homozygous null for GDF8 and GDF11 in muscle are phenotypically no different than GDF8-/- with regard to fiber numbers and size (McPherron et al., 2009). Thus, the identity of additional ActRIIb ligands remains unknown.
Bone Morphogenetic Protein 6

The majority of bone morphogenetic protein (BMP) effects on myogenesis are noted during embryogenesis (Buckingham et al., 2003). BMP2 and BMP4, secreted by the neural tube and notochord, serve to limit the size of the developing somitic myotome in chick embryos (Reshef et al., 1998). Inhibition of BMP activity by Noggin allows for expansion of the embryonic myoblast pool. However, neither BMP2 nor BMP4 is activated during muscle regeneration arguing that they play an insignificant role during postnatal muscle growth and repair (Zhao and Hoffman, 2004).

BMP6, originally named Vgr-1, is expressed by adult skeletal muscle (Lyons et al., 1989). Although BMP6-/- mice display no phenotypic abnormalities in muscle size or ambulatory function, the growth factor may act as a paracrine mediator of satellite cell activity (Solloway et al., 1998). BMP6 stimulates iNOS expression, a known mediator of satellite cell activation, in macrophages (Kwon et al., 2009). Moreover, treatment of C2C12 satellite cells with BMP6 suppresses MyoD expression and muscle fiber formation (Ouyang et al., 2006). It remains unclear if inhibition of myogenesis is a direct effect or a consequence of initiation of the osteogenic gene program and subsequent myoblast transdifferentiation (Ebisawa et al., 1999). An intriguing possibility is that BMP6 secreted by the muscle fiber serves as niche-localized factor that promotes satellite cell activation through NO production and suppresses precocious differentiation.

BMP6 regulatory effects are mediated through phosphorylation and activation of Smad1, Smad5 and Smad8, collectively referred to as Smad1/5/8, shortly after docking with an oligomerized BMP receptor (Valdimarsdottir et al., 2002). Unlike BMP2 and 4,
BMP6 exhibits high affinity binding with the type II receptor, activin receptor 2A or ALK2, prior to receptor oligomerization with BMPRI (Vukicevic and Grgurevic, 2009).

The unique nature of BMP6 further extends to co-receptor interactions. The receptor guidance molecule (RGM) family contains four members that serve as specific co-receptors for the BMPs (Corradini et al., 2009). RGMa, RGMb/DRAGON and RGMc/hemojuvelin (HJV) are expressed in mammals with RGMd found only in fish. RGMs are GPI-linked proteins with an extracellular ligand binding interface and no apparent cytosolic signaling motif. The three RGMs are expressed in several mouse tissues and are particularly abundant in skeletal muscle (Kanomata et al., 2009). RGMa is constitutively expressed during myogenesis and genetic ablation in mice causes neural tube defects with no apparent effect on skeletal muscle (Kanomata et al., 2009; Niederkofler et al., 2004). RGMb is up-regulated during C2C12 myoblast differentiation with ectopic expression inhibitory to BMP2 induced myoblast transdifferentiation (Kanomata et al., 2009). RGMb-/- mice are neonatal lethal with possible neural mapping defects (Mueller et al., 2006). RGMc increases dramatically prior to C2 myoblast fusion (Kuninger et al., 2006). However, forced expression of the putative signaling modulator neither promotes nor deters myofiber formation. RGMc-/- mice are viable with no discernible muscle defects but suffer juvenile hemochromatosis (Andriopoulos et al., 2009; Huang et al., 2005; Niederkofler et al., 2005). In a like manner, BMP6-/- animals accumulate extreme amounts of iron in the liver, pancreas and heart, a hallmark of juvenile hemochromatosis (Meynard et al., 2009). Infusion of soluble RGMc into mice effectively binds and inactivates BMP6 and induces iron accumulation in the serum and liver (Andriopoulos et al., 2009). Soluble RGMb bound BMP6 in vitro but failed to
interact with the growth factor in vivo. Thus, RGMc is a specific binding partner for BMP6, a critical mediator of iron homeostasis.

Since BMP6 is widely expressed in embryonic and adult tissue, including muscle, in many species, it is supposed that BMP6 is also expressed in bovine muscle. Thus BMP6 may have regulatory effects via SMAD pathway in bovine satellite cells, such as regulating proliferation, differentiation. Activated BSC can be divided into three groups based on Pax7 and Myf5 expression. Since the proportion of these groups is closely related to BSC lineage progression, BMP6 may also change the ratio of them. Moreover, BSC could be converted to osteogenic cells under the effect of BMP6 as the mouse myogenic cell, C2C12.
CHAPTER 2
MATERIALS AND METHODS

Bovine Satellite Cell Isolation

The semimembranosus muscle (≤455g) was harvested intact from Holstein bull calves (3-7 days of age) following euthanasia. Visible connective tissue was removed, and the muscle was finely minced with a commercial meat grinder. The tissue was incubated with 0.8 mg/ml Type XIV protease (Sigma, St Louis, MO) in Earle’s Balanced Salt Solution (EBSS; Sigma, St Louis, MO) for 1 hour at 37°C with gentle mixing at 10 minute intervals. The tissue slurry was centrifuged at 1500 X g for 10 minutes and the protease decanted. An equal volume of sterile phosphate buffered saline (PBS, pH 7.4) was added to the tissue and the slurry was vigorously shaken for 5 minutes to liberate the fiber-associated satellite cells. Cells were collected by centrifugation at 500 X g for 10 minutes and retention of the supernatant. The process was repeated for a total of 4 times. Cell pellets were collected by centrifugation at 1500 X g for 10 minutes, resuspended in growth medium (low glucose Dulbecco’s modified Eagle medium supplemented with 10% (v/v) horse serum (HS), 1% (v/v) 5000 Units/ml penicillin-streptomycin, 200 mM L-glutamine and 0.1% (v/v) 10mg/ml gentamicin). Cells were further purified by sequential filtration through 70 μm and 40 μm cell strainers (BD Falcon, Durham, NC). The resulting bovine satellite cells (BSC) were stored frozen in growth medium containing 10% (v/v) dimethyl sulfoxide in liquid nitrogen until use.

Cell Culture

All cell culture reagents were purchased from Invitrogen (Carlsbad, CA) unless otherwise noted. C2C12 mouse satellite cells and 23A2 embryonic myoblasts were cultured on 0.1% (w/v) gelatin coated tissue culture plasticware in high-glucose
Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin/streptomycin, L-glutamine, and 0.1% (v/v) gentamicin or basal Eagle medium (BME) containing 15% (v/v) FBS, 1% v/v penicillin/streptomycin, L-glutamine, and 0.1% (v/v) gentamicin, respectively. BSC were seeded at a density of 1.6X10^4/cm^2 on tissue cultureware coated with entactin-collagen IV-laminin cell attachment matrix (ECL) in high glucose DMEM containing 10% (v/v) horse serum (HS), 1% (v/v) penicillin/streptomycin, 1% (v/v) L-glutamine, and 0.1% (v/v) gentamicin reagent solution. Differentiation was induced by culture for 3 days in low glucose DMEM supplemented with 2% (v/v) HS, 1% (v/v) penicillin/streptomycin, and 0.1% (v/v) gentamycin. Where indicated, recombinant human BMP6 (R&D Systems, Minneapolis, MN) was supplemented at 50 ng/ml in growth or differentiation medium. Proliferation was measured by 5-ethynyl-2'-deoxyuridine (EdU), a nucleoside analog to thymidine, incorporation into DNA during the final 30 minutes or 2 hours of experimental treatment.

**Immunocytochemistry**

Myoblast cells were fixed with 4% (v/v) formaldehyde in PBS for 10 minutes at room temperature. Myofiber cultures were fixed with Alcohol-Formalin-Acetic Acid (AFA, 85% alcohol: 16% formaldehyde: 5% glacial acetic acid, v/v) for 15 minutes at room temperature. Fixed cells were washed thoroughly with PBS and non-specific binding sites were blocked with the blocking buffer (PBS containing 5% (v/v) HS and 0.1% (v/v) Triton X-100 (Fisher Scientific, NJ)) for 30 minutes at room temperature. Subsequently, cells were incubated with primary antibodies under the following conditions: mouse anti-Pax7 hybridoma supernatant (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), 1:10 in 0.1X Blocking Buffer, 4 C overnight; rabbit anti-Myf5 (C-20; Santa Cruz Biotechnology, Santa Cruz CA), 1:100 in 0.1X Blocking Buffer, 4 C
overnight; mouse anti-myosin heavy chain (MyHC) hybridoma supernatant (MF20; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), 1:20 in Blocking Buffer, room temperature, 1 hour. Primary antibodies were removed by washing with PBS (3 X 5 min). Immune complexes were detected with the appropriate anti-mouse AlexaFluor 488 and anti-rabbit AlexaFluor 527 (Invitrogen, Carlsbad, CA) diluted 1:200 in Blocking Buffer. Hoechst 33342 (5 μg/ml in PBS) was used to identify nuclei. After a final PBS wash, fluorescent-labeled complexes were visualized using an Eclipse TE 2000-U microscope (Nikon, Lewisville, TX) equipped with an X-Cite 120 epifluorescence illumination system (EXFO, Mississauga, Ontario, Canada). Photomicrographs were captured using a Photometrics Cool Snap EF digital camera (Nikon, Lewisville, TX).

**Alkaline Phosphatase Histology**

Semiconfluent myoblasts and BSC were cultured with 50 ng/ml BMP6 for 48 hours following fixation with 4% (v/v) formaldehyde for 10 minutes. After washing with PBS, the fixed cultures were incubated at 37 C for 18 hours with nitroblue tetrazolium and 5-bromo-4-chloro-3'-indolyphosphate p-toluidine (NBT-BCIP). Colorimetric alkaline phosphatase activity was visualized under bright field and phase microscopy. Representative images were captured with a DXM 1200F digital camera.

**Western Blots**

BSC, C2C12 and 23A2 myoblasts were treated with 10 μg/ml protamine sulfate (EMD Chemicals, Gibbstown, NJ) in serum free medium for 10 minutes at 37 C to remove surface associated growth factors. The cells were further incubated for one hour in serum free medium to reduce intracellular signaling events. BMP6 (50 ng/ml) was added to the medium and cells were lysed in 4X Lammeli buffer (250 mM Tris, pH 6.8,
8% (w/v) SDS, 40% (v/v) glycerol, and 0.4% (v/v) β-mercaptoethanol at the indicated times. Lysates were sonicated and heated for 5 minutes at 95 C. Total cellular protein from an equivalent number of cells was separated electrophoretically through 10% (v/v) polyacrylamide gels and transferred to nitrocellulose membrane. The membranes were incubated with 5% w/v nonfat dry milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% (v/v) Tween 20) for 30 minutes at room temperature to block non-specific antigen binding sites. Primary antibodies diluted in blocking solution were incubated with blots under the following conditions: rabbit anti-phospho-Smad1 (Ser463/465)/Smad5 (Ser463/465)/Smad8 (Ser426/428) (Cell Signaling Technology, Danvers, MA), 1:1000, 4 C overnight; rabbit anti-SMAD 1/5/8/9 (Abcam, Cambridge, MA), 1:1000, 4 C overnight; mouse anti-alpha tubulin (Abcam, Cambridge, MA), 1:5000, room temperature for 1 hour. After incubation, the blots were washed with TBST 3 times for 5 minutes each, then incubated with peroxidase-labeled anti-mouse or anti-rabbit antibody (Invitrogen, Carlsbad, CA) in blocking solution for 1 hour at room temperature. After washing with TBST, immune complexes were visualized with chemiluminescence and exposure to x-ray films (X-OMAT LS Scientific Imaging Films, Kodak, Rochester, NY).

**Luciferase Reporter Assay**

Cells were transiently transfected by DNA-calcium phosphate precipitate formation (C2C12, 23A2) or liposome-mediated DNA delivery (BSC; Lipofectamine 2000, Invitrogen, Carlsbad, CA). Plasmid DNA included BRE-Luc, a multimerized BMP response element driving luciferase expression, and pRL-tk, a plasmid coding for Renilla luciferase under control of a minimal thymide kinase promoter. The cells were cultured in the presence of transfection reagents for 5 hours. After 18 hours in growth medium, the cells were treated with 50 ng/ml BMP6 for 48 hours. Cells were lysed and...
luciferase activities were measured using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI).

**BMP6 RT-PCR**

Total RNA (1 μg) isolated from the semimembranosus of a young bull calf was digested with RNase-free DNase (Ambion, Austin, TX) for 30 minutes at 37 C prior to reverse transcription with 60 μM random hexamers (Promega, Madison, WI), 1 mM dNTP (Promega, Madison, WI), 40 units RNase inhibitor (New England Biolabs, Ipswich, MA), and M-MLV reverse transcriptase (200 units, New England Biolabs, Ipswich, MA). The resulting cDNA was amplified with bovine BMP6 forward (5’ TTGCCCCCAAGGGCTACGCT 3’) and reverse (5’ AGCACCGAGATGGCGTTCAGT 3’) primers and AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA) under the following conditions; 95 C 2 minutes, 40 cycles of 95 C 30 seconds, 65 C 30 seconds, and 72 C 40 seconds and a final extension step of 72 C 10 minutes. The BMP6 amplicon was visualized following electrophoresis through ethidium bromide impregnated agarose gels, extracted and sequenced on both strands.

**Statistics**

All data presented in this study represents at least three independent experiments with a minimum of two to three replicates per treatment group. All numerical data was analyzed with the PROC ANOVA procedure of the Statistical Analysis System (SAS, SAS inst. Inc., Cary, NC) where treatment, repeat, and their interaction were the fixed effects. Data was presented as Means ± standard error of the mean (SEM). Treatments were considered significantly different when P≤0.05.
CHAPTER 3
RESULTS

**BMP6 is Expressed in Bovine Skeletal Muscle**

*BMP6* is widely expressed throughout mouse embryonic and adult tissues including skeletal muscle (Lyons, 1989). In an analogous manner, *BMP6* is expressed in the newborn calf. Total RNA was isolated from the semimembranous muscle of a young Holstein bull calf (≤7 days) and analyzed by RT-PCR using gene specific primers (Figure 3-1). The resulting DNA amplification of 175 bp was sequenced on both strands. NCBI database search using the BLASTN engine revealed 100% homology to bovine *BMP6*. The gene is conserved with 45 orthologs present in genomes ranging from lizards (*Anolis carolinensis*) to dolphins (*Tursiops truncates*), fruitflies (*Drosophila melanogaster*) and humans (Figure 3-2). The extensive conservation of BMP6 suggests that it may participate in an integral developmental, metabolic or regulatory function.

**BMP6 Affects Distinct Aspects of Satellite Cell Myogenesis**

Members of the TGF-β superfamily, which includes BMP6, are noted inhibitors of satellite cell and myoblast proliferation and differentiation. The effects of BMP6 on bovine satellite cell (BSC) proliferation and myofiber formation were examined. BSCs were isolated, seeded on gelatin-coated tissueware and cultured in growth medium for 48 hours. Subsequently, the medium was replaced with low-serum medium supplemented with 50 ng/ml BMP6 or vehicle for 48 hours. Embryonic mouse myoblasts (23A2) and mouse satellite cells (C2C12) were treated in an analogous manner. All cells were pulsed with the thymidine analog, EdU, prior to fixation. The numbers of cells that incorporated EdU were measured and expressed as a percent of total cells (Figure
A modest decline (~5%) in cell proliferation was apparent for all myogenic cell types treated with BMP6.

The limited response suggests that muscle-derived BMP6 does not serve as a primary mediator of satellite cell quiescence and proliferative activity. Although no robust change in total cell numbers were observed, BMP6 may affect satellite cell lineage progression. Satellite cells are a heterogeneous population comprised of both muscle stem and progenitor cells. Muscle stem cells, defined by Pax7 expression, give rise to myoblast progenitors that express both Pax7 and Myf5 (Buckingham, 2007). The effects of BMP6 on the two subpopulations were examined following 48 hrs of treatment (Figure 3-4). In brief, BSC were cultured in growth permissive medium supplemented with 50 ng/ml BMP6 for 48 hours followed by fixation and immunodetection of Pax7 and Myf5. The numbers of Pax7-only, Pax7+/Myf5+ and Myf5-only cells were measured and expressed as a percentage of total cells. BMP6 did not affect the percent of Pax7-only muscle stem cells. However, a reduction in the percent of Pax7+/Myf5+ progenitors and an increase in the numbers of Myf5-only myoblasts were evident. The population shift from progenitor to committed myoblast indicates that BMP6 promotes satellite cell myogenesis independent of an effect on global proliferation rate.

The ability of BMP6 to accelerate the transition from progenitor to myoblast also may hasten myofiber formation. BSC cultures were placed in differentiation permissive medium supplemented with 50 ng/ml BMP6. 23A2 and C2C12 myogenic cells were treated in a similar manner. After 48 or 72 hours, the cells were fixed and immunostained for myosin heavy chain (MyHC). Total nuclei were visualized with Hoechst 33245. As shown in Figure 3-5A, BSC readily form large myosin-expressing,
multinucleated fibers *in vitro*. Many of these structures contain over 100 nuclei. A substantial reduction in the myofibers was noted following BMP6 treatment. Enumeration of myofiber nuclei revealed an approximate 50% reduction in differentiation in response to growth factor treatment (Figure 3-6). These results demonstrate that BMP6 exerts strikingly different effects on satellite cell myogenesis by promoting myoblast pool expansion and suppression of myofiber formation. A similar 50% reduction in myofiber formation was observed for 23A2 myoblasts treated with BMP6 (Figure 3-5B). By contrast, a dramatic inhibitory effect was noted for C2C12 satellite cells treated with BMP6 (Figure 3-5C). Less than 1% of the total nuclei were contained within MyHC immunopositive cells.

**Repression of BSC Myogenesis is Independent of Transdifferentiation**

C2C12 myoblasts are notably responsive to BMP2, 4 and 6 whereby they undergo transdifferentiation to an osteogenic phenotype (Yamamoto, 1997, Li, 2005, Ebisawa, 1999). The ability of BMP6 to block BSC differentiation by initiation of the osteogenic gene program was examined. In brief, BSC, 23A2 and C2C12 myogenic cells were treated for 48 hours with BMP6 followed by fixation and histological staining for alkaline phosphatase activity. As expected, C2C12 myoblasts readily adopted the osteogenic phenotype as indicated by strong AP activity. By contrast, neither 23A2 nor BSC expressed the bone enzyme in response to the growth factor (Figure 3-7).

The inability of the cells to convert to the osteogenic lineage is not due to a defective SMAD1/5/8 signaling pathway. Subconfluent cultures were serum-deprived for one hour followed by treatment with BMP6. Cells were lysed and evaluated by Western blot for total and phosphorylated SMAD content. BSC activate the SMAD signaling axis within 5 minutes of BMP6 treatment and exhibit maximal, sustained activity within 30
minutes (Figure 3-8A). Both 23A2 and C2C12 myoblasts demonstrate abundant amounts of phosphorylated SMAD1/5/8 (Figure 3-8B, C).

The ability of the myogenic cells to activate SMADs and elicit a transcriptional response was examined using a BMP-response element reporter gene (BRE-Luc). The myogenic cells were transiently transfected with BRE-Luc and pRLtk-Luc, a transfection efficiency monitor, prior to treatment with BMP6. After 48 hours, the cells were lysed and luciferase activities measured. All three myogenic cells directed transcription from the BMP-response reporter gene, although at differing levels (Figure 3-9). By comparison to 23A2 and C2C12 myoblasts, BSC direct higher levels of basal BRE-Luc transcription. These results demonstrate that the SMADs are phosphorylated in response to BMP6, and translocate to the nucleus to initiate transcriptional change.
Figure 3-1. Bovine muscle tissue expresses BMP6. RT-PCR was performed on bovine semimembranosus total RNA using primers specific for bovine BMP6 and GAPDH transcripts. Both transcripts were expressed. Representative photo is shown.
Figure 3-2. BMP6 phylogenetic tree (Source: http://uswest.ensembl.org/Bos_taurus/Gene/Compara_Tree?db=core;g=ENSBTAG00000019234;r=23:48406821-48416543;t=ENSBTAT00000025614. Last accessed May, 2010)
Figure 3-3. BMP6 inhibits EdU incorporation. BSC (pre-cultured for 24 hours), 23A2 and C2C12 myoblasts were treated with 50 ng/ml BMP6 for 48 hours and pulsed with EdU for the last 30 minutes (23A2 and C2C12) or 2 hours (BSC). Cells were fixed and immunostained to quantify the percent of cells that incorporated EdU. All experiments were repeated 3 times. Error bars indicate SEM. Asterisks indicate significant difference, p<0.05 (*) or p<0.0001 (**).
Figure 3-4. BMP6 changes BSC subpopulation proportions. BSC were cultured with 50 ng/ml BMP6 for 48 hours. Cells were fixed and immunostained for Pax7 and Myf5. Hoescht dye was used to identify nuclei. The proportion of different BSC subpopulations in response to treatment was quantified. All experiments were repeated 3 times. Error bars indicate standard error of the mean (SEM). Asterisks indicates significant difference, p<0.05 (*) or p<0.0001 (**).
Figure 3-5. BMP6 inhibits myofiber formation. BSC (pre-cultured for 3 days), 23A2, and C2C12 were cultured with 50 ng/ml BMP6 or vehicle-only for 48 or 72 hours. Cells were fixed and immunostained for myosin heavy chain (MyHC). Total nuclei were identified with Hoechst 33245. A) BSC myofiber formation was inhibited by BMP6. B) 23A2 myofiber formation was repressed by BMP6. C) C2C12 myofiber formation was restrained by BMP6.
Figure 3-5. Continued.
Figure 3-6. BMP6 inhibits differentiation of BSC and myoblasts. BSC (pre-cultured for 3 days), 23A2 and C2C12 were cultured with 50 ng/ml BMP6 or vehicle-only for 48 hours (BSC) or 72 hours (23A2 and C2C12). Cells were fixed and immunostained for myosin heavy chain (MyHC). Total nuclei were identified with Hoechst staining. Fusion index was calculated as numbers of nuclei in MyHC positive fibers (containing at least 3 nuclei) divided by number of total nuclei. BMP6 suppressed differentiation and fusion in all three types of cells. All experiments were repeated 3 times. Error bars indicate SEM. Asterisks indicate significant difference, p<0.05 (*) or p<0.0001 (**).
Figure 3-7. BMP6 induces alkaline phosphatase (ALP) activity in C2C12 myoblasts but not 23A2 myoblasts and BSC. BSC, C2C12 and 23A2 myoblasts were treated with 50 ng/ml BMP6 for 48 hours. Cells were fixed and ALP activity was measured by colorimetrical reaction with NBT/BCIP. Representative photomicrographs at 100X are shown. A) BMP6 induced ALP activity in C2C12 myoblasts. B) BMP6 did not induced ALP activity in 23A2 myoblasts. C) BMP6 did not induced ALP activity in BSC.
Figure 3-7. Continued.
Figure 3-8. BMP6 activates SMAD1/5/8 in BSC, 23A2 and C2C12 myoblasts. A) BSC were treated with 50 ng/ml BMP6 for 0, 5 minutes, 30 minutes and 1 hour after serum-starvation. Total protein isolates were harvested and analyzed by Western blot for SMAD1/5/8/9, phosphorylate SMAD1/5/8 or tubulin protein expression. B) 23A2 myoblasts were treated with 50 ng/ml BMP6 for 0 and 30 minutes after serum-starvation. Total protein isolates were harvested and analyzed by Western blot for SMAD1/5/8/9, active SMAD1/5/8 or tubulin protein expression. C) C2C12 myoblasts were treated with 50 ng/ml BMP6 for 0 and 30 minutes after serum-starvation. Total protein isolates were harvested and analyzed by Western blot for SMAD1/5/8/9, active SMAD1/5/8 or tubulin protein expression.
Figure 3-8. Continued.
Figure 3-9. BMP6 stimulates transcription of BRE-Luc. BSC (pre-cultured for 3 days), 23A2 and C2C12 myoblasts were transfected with BRE-Luc reporter and pRL-tk. Cells were treated with 50 ng/ml BMP6 for 48 hours. Cell lysis were harvested and analyzed using a Dual-Luciferase Reporter Assay System. Reporter luciferase activity was normalized to the amount of Renilla luciferase activity. All experiments were repeated 3 times. Error bars indicate SEM. Asterisks indicate significant difference, p<0.05 (*) or p<0.0001 (**).
CHAPTER 4
DISCUSSION

Bone morphogenetic protein (BMP6) belongs to transforming growth factor beta (TGF-β) super family which contains many important growth factors involved in myogenesis regulation. Previous researches display wide expression of BMP6 in embryonic and adult tissue, including skeletal muscle. It is documented that BMP6 can convert a myogenic cell line, C2C12, to osteogenic cells (Fujii et al., 1999). And also, BMP6 can stimulates iNOS expression, a known mediator of satellite cell activation, in macrophages (Kwon et al., 2009). Thus, BMP6 is indicated a potential role as a niche-localized regulatory factor of satellite cells.

To characterize the regulatory effects of BMP6 in satellite cells, especially in bovine satellite cell. Three types of cells were used in this study: two commonly used mouse satellite cell models, C2C12 and 23A2 cell lines, and an bovine in vitro satellite cell model, BSC.

First of all, the endogenous BMP6 expression in immature bovine muscle tissue was demonstrated by the RT-PCR. The expression of BMP6 indicates potential regulation effects of BMP6 in bovine muscle. To determine the effects, Three different types of muscle cells were used: BSC, 23A2 myoblast, and C2C12 myoblast.

It was noticed that BMP6 inhibited proliferation of all three cell types; however, the decline in proliferation was not large enough to conclude that BMP6 plays the main role in mediating satellite cell quiescence and proliferative activity. Although no notable change in total cell numbers were observed, BMP6 affects satellite cell lineage progression. As Figure 4-1 shows, quiescent satellite cells are a heterogeneous population comprised of both muscle stem and progenitor cells. Once they are
activated, Pax7-only muscle stem cells can not only selfrenew but also give rise to committed myo-progenitors which express both Pax7 and Myf5. Pax7+/Myf5+ myo-progenitors can become Myf5-only myoblasts during myogenesis. In BSC cultures, cells were already activated and there were three main subpopulations: Pax7-only muscle stem cells, Pax7+/Myf5+ muscle progenitors, and Myf5-only myoblasts. BMP6 did not significantly affect the percent of cells that were muscle stem cells. However, the percent of Pax7+/Myf5+ progenitors decreased and the percent of Myf5-only myoblasts increased. The population shift from progenitor to myoblast indicates that BMP6 promotes satellite cells myogenesis independent of an effect on global proliferation rate.

Nevertheless, BMP6 does not accelerate myofiber formation. In contrast, a substantial reduction in the myofibers was noted following BMP6 treatment on BSC. A similar reduction in myofiber formation was observed in 23A2 treated with BMP6, also a dramatic inhibitory effect was noted in BMP6 treated C2C12. These results demonstrate that BMP6 exerts strikingly different effects on satellite cell myogenesis by promoting myoblast pool expansion and suppression of myofiber formation.

It is documented that BMP6 can induce many different cells in vivo (Gitelman, 1994) or in vitro (Gruber, 2003; Ouyang, 2006; Estes, 2006) to endochondral bone pathway in previous studies. C2C12 myoblasts in particular are notably responsive to BMP2, 4 and 6 whereby they undergo trans-differentiation to an osteogenic phenotype (Yamamoto, 1997, Li, 2005, Ebisawa, 199). Strong ALP activity was observed in C2C12 treated with BMP6 in the study as expected, which indicated the osteogenic trans-differentiation. Interestingly, neither 23A2 nor BSC expressed the bone enzyme in response to the growth factor. This shows that the repression of BSC myogenesis
differentiation was not because of osteogenic trans-differentiation. Also the inability of the cells to be converted to the osteogenic lineage is not due to a defective SMAD1/5/8 signaling. In all three cell types, BMP6 activated SMAD1/5/8, and trigger a transcriptional level response in nuclei.

Thus, the intracellular pathway of BMP6 in BSC is intact. BMP6 regulates BSC through SMAD pathway and in two distinct aspects: the proliferation and the myogenic differentiation are inhibited by BMP6, which are negative regulatory effect of myogenesis; relatively more muscle progenitors were converted into myoblasts under the effect of BMP6, which is positive regulatory effect of myogenesis. But BMP6 fails to convert myogenic differentiation to osteogenic differentiation in BSC.

The different responses to BMP6 between BSC and other cells does not simply relate to species difference. BSC, 23A2, and C2C12 are all in vitro satellite cell model. BSC are primary cells, so they can better mimic the in vivo situation. 23A2 myoblasts were induced from a mouse embryonic cell line C3H10T1/2 (Konieczny and Emerson, 1984) and C2C12 myoblasts were isolated from dystrophic mouse muscle (Yaffe and Saxel, 1977). They are both mouse cells. Regarding to differentiation, the response modes to BMP6 of 23A2 and BSC are more alike than C2C12. Although the differences of their intracellular pathways are not yet clear, it seems that 23A2 may be a better mice mouse model to use in comparative studies with BSC, especially studies relating to BMP6.

BSC is an important in vitro satellite cell model in muscle studies. Because of the bigger muscle size and larger satellite cell amount of bovine than other commonly used animals, it is more efficient to obtain satellite cells from bovine, which is benefit for
repeating studies in one animal or certain muscle if necessary. Some breeds have natural mutation of genes, such as Belgium Blue and Piedmontese cattle, which have GDF8 mutation and are known as double muscling (Kambadur, 1997). So satellite cells from those breeds are good to be used in studies of those genes and their interaction of other genes. Also, cattle are an important kind of meat-producing animal. An understanding of muscle biology in bovine can help improve production efficiency in the cattle industry. Muscle contains abundant storage iron. Iron overload can cause hemochromatosis, while iron deficiency can convert the red Fast Oxidative-Glycolytic (FOG) muscle fibers into white Fast Glycolytic (FG) fibers, which will decline the meat tenderness (Gordeuk et al., 1987; Klont et al., 1998; Ohira and Gill, 1983). Since BMP6 is a critical mediator of iron homeostasis (Andriopoulos et al., 2009), it is indicated that the expression of BMP6 is related to meat quality. If the mechanism behind can be found out, it may promote meat producing by optimizing breeding or feeding.

This work revealed some regulatory effects of BMP6 in BSC, 23A2 and C2C12, but still left some unknown and unexplored. The BMP inhibitor, Noggin, or small interfering RNA (siRNA) can be applied to block BMP6 effects and observe the responses of the cells. To find out the different response mechanism of BSC and 23A2 from C2C12, some factors in the signaling pathway, such as RGMs, need to be considered in the future work. Also the in vivo responses of satellite cells to BMP6 should be studied later.
Figure 4-1. Illustration of satellite cell subpopulations and myogenesis. (Modified from Winata and Gerace's figure in http://www.bio.purdue.edu/people/faculty/konieczny/lab/MyoDresearch.htm)
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Wenli Sun was born in Shanghai, China, to Yongxian Sun and Caizhen Pan. She grew up as the only child of this family and completed all of her educations until university in this large city. In July 2006, Wenli Sun graduated with a bachelor’s degree in veterinary medicine from Shanghai JiaoTong University. She then moved to the United States and began study at the University of Florida under the advisement of Dr. Sally Johnson in 2007. Wenli Sun currently resides in Gainesville, Florida with Tony the fish and Shadow the cat. Upon receiving the master’s degree, Wenli Sun will go back to her hometown and stay with her families. She hopes to find a position in related area there, continue exploring the sea of knowledge, and ultimately find her interest.