INVOLVEMENT OF BMP6 AND E2F5 IN SKELETAL MYOGENESIS

By

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To Bill and my family for all of their love and support. I would be lost without you guys! All of my love, J.

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An amazing dynamic exists within skeletal muscle that is required for the development and maintenance of the musculature system in response to stimuli. A differential BMP- and TGFβ-responsiveness and gene expression profile in myoblasts versus myofibers, suggests that TGFβ superfamily members play distinct regulatory roles at specific stages of myogenesis. Treatment of myoblasts with BMP6 results in an increase in alkaline phosphatase activity in a dose-dependent and time-dependent manner. Exogenous BMP6 treatment results in inhibition of myoblast differentiation as observed by significant inhibition of muscle reporter activity, muscle specific protein synthesis, and myoblast fusion. BMP6 treatment does not alter proliferation rates or play an anti-apoptotic role in myoblasts. Inhibition of p38 activity and BMP6 treatment caused significant inhibition of TnI-Luc activity and muscle specific protein expression markers suggesting an additive effect through independent signaling cascades. Interestingly, the combination of BMP6 and Notch inhibition partially restores MyHC expression in fibers. This suggests that the myogenic inhibitory effect observed in the presence of BMP6 is not a cell cycle effect and not a direct target of the Raf/MEK/ERK signaling axis, but is partially mediated by functional Notch signaling. Therefore, BMP6-mediated inhibition of differentiation is regulated or partially controlled by the Notch signaling pathway. Ectopic BMP6 treatment did not induce

E2F5 nuclear accumulation in myoblasts further suggesting BMP6 is not causing a cell cycle effect. E2F5 is present in non-dividing cells *in vivo* outside the dystrophin border in the putative satellite cell position, although E2F5 did not impact myogenic differentiation. Additionally, the inability of E2F5 to inhibit differentiation is not due to insufficient pocket protein function. Multiple signaling axes are key factors during Raf-imposed block to myogenic differentiation. Further understanding of the regenerative ability in skeletal muscle in response to stimuli would be useful for patients or animals that experience severe muscle trauma, and for individuals with skeletal muscle disorders, and would provide additional information for human therapeutic and agricultural applications that would benefit from enhanced muscle mass.

CHAPTER 1
LITERATURE REVIEW

Introduction

Within an organism, skeletal muscle functions in locomotor activity, postural behavior, and breathing. Skeletal muscle will undergo injury in response to direct trauma or as an indirect result of neurological dysfunction or genetic defects, and if not repaired, muscle mass and locomotion abilities may be lost, and death can possibly occur (Chargé and Rudnicki, 2004). An amazing dynamic exists within skeletal muscle that is required for the maintenance of the musculature in regards to the states of atrophy, injury, and subsequent repair. Understanding the regenerative ability of skeletal muscle in response to these stimuli are important areas of research. For instance, patients who experience severe muscle trauma, individuals with genetic skeletal muscle disorders like muscular dystrophy, and improving the recovery of astronauts experiencing a weightless environment would benefit from such studies. Many changes to muscle physiology occur in an adaptive response to decreased or increased usage of skeletal muscle (Stein and Wade, 2005). The adaptive responses of decreased usage includes a shift in myosin isoforms from slow to fast fiber type, replacement of protein with fat within the muscle, shifts in energy source for metabolism away from lipids to glucose, a loss of bone mineral density, and an increase in bone resorption (Shackelford et al., 2004; Stein and Wade, 2005).

Skeletal Muscle Biology

The mechanics of skeletal muscle contraction begin with a neural stimulus for contraction. This results in a generation of an action potential in a motor neuron causing the release of acetylcholine into the synaptic cleft of the neuromuscular junction (NMJ). This released acetylcholine binds with the receptors on the motor end-plate, producing an end plate potential, which leads to the depolarization that is conducted down the transverse tubules deep into the

muscle fiber. Depolarization results in calcium being released from the sarcoplasmic reticulum (SR) (Crouch, 1985).

In the resting state, myosin cross-bridges remain connected to actin in a weak binding state. When depolarization signal reaches the SR, calcium is released into the sarcoplasm, which binds to troponin, causing a shift in troponin position to uncover the "active" sites on actin. The "energized" or "cocked" myosin cross-bridge then forms a strong bond at the active sites on actin. Inorganic phosphate released from the myosin cross-bridge energizes the cross-bridge to allow it the ability to pull the actin molecules. Cross-bridge movement is completed by the release of ADP from the myosin cross bridge. At this point, the myosin cross-bridges are in what is considered the "strong binding state" with actin. Attachment of ATP to myosin allows the cross-bridge to break the strong binding state and form a "weak binding state". ATP is then broken down to ADP + Pi + energy. The energy is released and used to "energize" the myosin cross-bridges. This cycle can repeat as long as calcium and ATP are present. The cycle is stopped when the SR actively removes calcium from the sarcoplasm (Lieber, 2002).

The SR and Transverse Tubule Systems within Skeletal Muscle

Within the muscle, two membrane systems are present to activate the filaments. These two systems are called the transverse tubule (T) system and the SR system. The T-system consists of invaginations in the surface membrane physically contiguous with the sarcolemma, or holes that extend into the fiber and transversely crosses the long axis of the fiber. The T-system conveys activation signals received from the motor neuron into the myofibrils. Since motor neurons are not in direct contact, the T-system helps increase activation time versus a simple diffusion system. The release of calcium switches on muscle activation and the removal of calcium from the myofilament cause muscle relaxation. Embedded in the SR membrane are specific calcium channels and pumps that control this calcium release and uptake. The SR envelops each

myofibril to provide contact between the activation and force generation systems. The SR also contacts the T-system, thus acting as a middle man in skeletal muscle activation and relaxation. The T and SR systems are arranged in a structure called a "triad", which is a single T tubule surrounded by two SR tubules located at each Z disk and the triad functions as the interface between the extracellular and intracellular surfaces of the fiber (Lieber, 2002).

Additionally, the SR and T systems are linked by junctional feet structures. Junctional feet are composed primarily of two components: ryanodine receptors (RyR) and dihydropyridine receptors (DHP). RyRs are voltage sensing proteins embedded in the SR that sense action potential traveling across the T system. DHPs are calcium release channels through which the myofilament receives activating calcium (Lieber, 2002).

Major Fiber Type Classification

Skeletal muscles can be classified as either red or white based on the major proportion of red or white fibers they contain. Few muscles are composed solely of one fiber type; most are of a heterogeneous composition. The red coloration of red fibers is due to higher myoglobin content as compared to white fibers and the myoglobin allows for oxygen storage required for oxidative metabolism. Conversely, white fibers have a high content of glycolytic enzymes and low levels of oxidative enzyme activity (Smith, 1972).

Another method of skeletal muscle fiber designation is based upon contraction rates: slow-twitch vs. fast-twitch fibers or type I vs. type II. There are four different adult myosin isoforms in skeletal muscle: type I, IIA, IIX(D), and IIB with type I and IIA designated as red muscle fibers and type IIX(D) and IIB designated as white muscle fibers (Bárány, 1967; Roy et al., 1984). Slow-twitch fibers, which are also called Type I fibers, are characterized by higher number of mitochondria, larger mitochondria, and the mitochondria are located in two general areas: the subsarcolemma, and the intermyofibrillar. The mitochondria within Type I fibers are

more efficient than in Type II fibers. For example, in the subsarcolemma, the mitochondria are nine times more efficient in Type I and in the myofibrillar, they are five times more efficient in I. These fibers are surrounded by numerous capillaries and have a higher concentration of myoglobin, which gives the red muscle fiber types their visual color. Type I fibers have a large capacity for aerobic metabolism and can use ATP more efficiently than Type II without lactic acid buildup. The lipid content is greater in red muscle fibers, which serves as a metabolic fuel source and while these fibers contract slower, they can contract for a longer time. These fibers play a role in posture because they are less easily fatigued, as long as oxygen is available and due to their higher resistance to fatigue and allowance for a higher maximal oxygen uptake (VO_2 max) they are selected for in endurance type training (Evans et al., 1994; Van Swearingen and Lance-Jones, 1995; Lieber, 2002).

Fast twitch or Type II fibers are glycolytic and can produce force at a higher rate. This is called a phasic mode of action since contraction occurs in short bursts and these fibers are more easily fatigued. White fibers have a more extensively developed SR and T-tubule system along with more narrow Z disks versus white fibers. They also have fewer and smaller mitochondria than Type I and the glycolytic metabolism that predominates in these fibers can occur both aerobically or anaerobically. White fibers have a lower capillary density than red fibers. Functional implications demonstrate that Type II fibers are increased during strength training due to their ability to produce force at a higher rate (Zhang and McLennan, 1998; Lieber, 2002).

Muscle Architecture

The anatomical and biomechanical properties of skeletal muscle determine the efficiency and overall ability of the muscle to generate force and movement. The largest functional unit of contractile filaments is the myofibril, which is a string of sarcomeres. The muscle fiber is

composed of myofibrils arranged side-by-side and the groups of muscle fibers are sheathed in connective tissue termed the perimysium (Lieber, 2002).

The number of sarcomeres within a fiber depends on the muscle fiber length and diameter of the fiber. They are the most important determinant of muscle fiber function. The total distance of myofibrillar shortening is equal to the sum of the shortening distance of each sarcomere. This allows for whole muscles to be able to shorten up to several centimeters even though each sarcomere can only shorten ~1 μm giving the muscle a tremendous ability to adapt. Within the sarcomere are two major types of contractile filaments, the thick filaments and the thin filaments. These filaments are large polymers of myosin and actin proteins. In thick filaments, which are the myosin-containing filament, a "feathered" appearance is observed with projections coming out at either end of the filament because one molecule rotates ~60° relative to molecules on either side. These filaments generate the tension during muscle contraction and the thin filaments or actin-containing filament regulates the tension generated (Gordon et al., 1966a; Gordon et al., 1996b; Lieber, 2002). Tension generation is also a function of the magnitude of the overlap between the actin and myosin filaments. Passive tension plays a role in providing resistance in the absence of muscle activation. The source of passive tension is due to the protein titin, which connects the thick myosin filaments end to end (Magid and Reedy, 1980; Labeit and Kolmerer, 1995).

Striated skeletal muscle is composed of bundles of enormous, multi-nucleated cells and the striations arise from a repeating pattern in the myofibrils. Active interdigitations of these filaments produce muscle shortening and give muscle its striated appearance. Some characteristics of striated muscle are its ability to contract and generate tension when activated and its ability to return to its original length and form after contraction or stretching ceases.

These properties are primarily the result of separate sets of filament systems: contractile actin and myosin filaments and viscoelastic titin and intermediate filaments (Vigoreaux, 1994; Knupp et al., 2002).

Within the thin filaments, approximately 5% of the myofibrillar protein is composed of troponin molecules, which are located approximately every seven or eight actin molecules along the actin filament. Troponins are the regulatory proteins responsible for turning on contraction. Troponin is composed of three subunits: Tn-I, Tn-C, and Tn-T. Tn-I exerts an inhibitory influence on tropomyosin when calcium is not present. Tn-C: binds calcium during contraction. Tn-T: binds troponin to tropomyosin. Tropomyosin is a long, rigid, and insoluble rod-shaped molecule that stretches along in close contact with each strand of the thin filament. Within each groove of the actin super-helix lays a strand of tropomyosin and a single molecule extends the length of seven G-actin molecules (Lieber, 2002).

The dark striations observed in skeletal muscle are a lattice of thick filaments termed the A band because it appears dark under a microscope. The light striations are termed I bands, which are regions of the myofibril containing only thin filaments and Z disks. The Z disks are the structure that attach to the thin filaments to act as actin-anchoring structures and function as a common link to mechanically integrate contractile and elastic elements (Schroeter et al., 1996). They are also involved in transmission of active and passive forces and differences in Z band structure have been described for distinct classes of muscle and fiber types (Vigoreaux, 1994). Thin filaments extend from two adjacent Z lines to interdigitate with the thick filaments. Thick and thin filament arrays form the contractile system and are the repeating unit in each myofibril (Bloom and Fawcett, 1968).

Intermediate Filaments (IFs) are one of the three major classes of cytoskeletal proteins along with microtubules and microfilaments. The IF proteins and IF-associated proteins such as desmin, vimentin, and nestin, are involved in maintaining the structural and functional integrity of all muscle types and align themselves in a head-to-tail dimer along the central rod domain. For example, in the skeletal muscle injury model, nestin and vimentin IF proteins begin to be expressed 6 hours post-injury in myoblasts and their maximal expression was observed around 3-5 days post injury. Thereafter, vimentin expression ceases completely, whereas nestin is found to remain only in the sarcoplasm next to neuromuscular and myotendinous junctions. At 6-12 hours post-injury, desmin expression is upregulated and becomes the predominant IF protein in myofibers, coinciding with the appearance of cross-striations. Nestin and vimentin are essential during the early phases of myofiber regeneration and desmin is responsible for maintenance of myofibrils in mature myofibers (Vaittinen et al., 2001).

A protofibril is a complex of eight IF monomers and the network of IFs form a lattice of connections that link the different parts of the muscle fiber. These networks extend from the Z-disk to adjacent myofibrils and from the sarcolemma to the Z-disc. Additionally, IFs are associated with the neuromuscular junction, nuclear membrane, and mitochondria located between adjacent myofibrils (Agbulut et al., 2001; Reipert et al., 1999).

Skeletal Muscle Function

Contraction velocity is based on the efficiency of the energy metabolism. Velocity is fastest when ATP is generated from an oxidative rather than a glycolytic pathway. In addition, contraction velocity is also dependent on the type of myosin present in the fiber. The four Myosin Heavy Chain (MyHC) isoforms are the products of a multigene family whose expression during development and in the adult are regulated by neuronal, hormonal, and mechanical factors. Most single fibers (~86%) express only one MyHC isoform but there are examples of

co-expression of MyHC isoforms and the MyHC IIA and IIX/D isoforms are predominately expressed among fibers (Rivero et al., 1999). In terms of shortening velocity, the gradient observed for MyHC isoforms is I < IIA < IIX/D < IIB (Bottinelli et al., 1991; Galler et al., 1994).

There are also phenotypic differences associated with MHC isoform composition in terms of metabolic and size properties of the muscle fiber types (Bär and Pette, 1988; Schiaffino et al., 1989). For example, motor units containing more MyHC I or IIA are more resistant to fatigue than motor units containing MyHC IIX/D or IIB. Additionally, MHC IIB fibers are generally larger than MyHC I or IIA (Rivero et al., 1999).

The globular head on the myosin has ATPase activity. This activity determines how fast the muscles contract, and is determined by how quickly ATP is hydrolyzed. In terms of the force-velocity relationship, this illustrates that the amount of force generated by a muscle is highly proportional to its velocity or velocity is dependent on how much force is resisting the muscle. Maximum contraction velocity is termed V_{max}, which is one of the most commonly used parameters to characterize muscle, and is relative to both fiber type distribution and architecture (Lieber, 2002).

Function is largely determined by a muscle's architecture. While fiber size between muscles does not vary greatly, there are major architectural differences between different muscle groups and these are the best predictors of force generation (Burkholder et al., 1994). Skeletal muscle architecture is defined as the arrangement of muscle fibers within the muscle relative to the axis of force generation. There are three classifications of muscle arrangements termed parallel, uni-pennate, and multi-pennate. Parallel muscles are composed of fibers that extend parallel to the muscle's force-generating axis. Uni-pennate muscles have fibers that are orientated at a single angle relative to the force-generating axis typically in an angle of 0 to

30 degrees. Most muscles are multi-pennate, where the fibers are orientated at several angles relative to the axis of force generation (Lieber, 2002). Other parameters that help characterize the properties of various muscles or muscle groups are the sarcomere length, which also translates into fiber length, range of movement (ROM), and the physiological cross-sectional area (PCSA). The PCSA of a muscle represents the sums of the cross-sectional areas of all of the muscle fibers within the muscle and is directly proportional to the maximum titanic tension generated by the muscle (Powell et al., 1984).

Tying together some of these parameters give a better indication of functional properties of specific muscle groups. For example, the quadriceps muscles have relatively high pennation angles, large PCSAs, and short fibers. Therefore, this makes them suited for the generation of large forces. Hamstring muscles like the sartorious, semitendinosus, and the gracilis muscles have low PCSAs and extremely long fibers, which allow for large excursions at low forces. Conversely, the soleus muscles have high PCSAs and short fiber lengths so that these muscles can generate high forces with small excursions making these suitable muscle groups for a postural stabilization role (Edman et al., 1985; Woittiez et al., 1985).

There is also different terminology related to different types of contractions. For example, isometric or static contractions are when the muscle tension increases but the body part does not move. Concentric contractions are when the action of the muscle results in muscular shortening as the body part moves and the force is applied to the muscle as it contracts. Eccentric contractions occur when the muscle is activated and force is produced as the muscle lengthens. Therefore, these types of contractions are more likely to result in muscle injury or soreness versus concentric contractions. Additionally, muscle strengthening regimens are greatest when exercises using eccentric contractions are utilized (Lieber and Friden, 2002). These factors

demonstrate why muscle architecture is also a consideration in training regimes for human and animal athletes. As more information is generated on the basic understanding of muscle form and function, there can be further benefits for athletic training and performance.

Skeletal Muscle Development

Skeletal muscle development is a highly complex and orchestrated process, which is still not completely understood, both at the macroscopic and molecular levels. Skeletal muscle cells are derived from the somites, except for the craniofacial muscles in mammals and birds (Armand et al., 1983). Somites are balls of epithelial cells formed from the paraxial mesoderm in pairs on either side of the neural tube, and differentiate into two regions - the dermomyotome and sclerotome. Due to their interactions with surrounding tissues, the ventromedial portion of the somite gives rise to the sclerotome, which is the precursor to the ribs and axial skeleton, and the dorso-lateral part forms the dermomyotome, which is where the myogenic precursors and dermis originate in embryogenesis (Braun et al., 1992; Rudnicki et al., 1993; Christ and Ordahl, 1995). Within the dermomyotome, the epaxial muscles and subsequent body wall muscles are formed on the dorso-medial lip. The hypaxial muscles, which consist of the limbs, tongue, diaphragm and ventral wall musculature, are formed on the dorso-lateral lip. Endothelial precursors are also derived from the somites (Chevallier et al., 1977; Beddington and Martin, 1989; Ordahl and LeDouarin, 1992; Wilting et al., 1995; Kardon et al., 2002).

Multiple pathways mediate the embryonic processes throughout skeletal muscle formation. In terms of limb bud development, the progenitors of this process evolve by delamination and migration distally into the developing limb bud in response to molecular signals from the adjacent lateral plate mesoderm (Chevallier et al., 1977; Christ et al., 1977; Solursh et al., 1987; Hayashi and Ozawa, 1995). Induction of premyogenic cells in the limb to delaminate is mediated by hepatocyte growth factor (HGF) or scatter factor and by fibroblast growth factor

(FGF). In experiments where both FGF2 and HGF were applied ectopically in the interlimb flank mesenchyme of chicks, delamination of the lateral dermomyotome was observed (Brand-Saberi et la., 1996; Heymann et al., 1996). Additionally, when c-met, the tyrosine kinase receptor for HGF, is inactivated, delamination in mice is prevented. Both HGF and FGF2 and FGF4 are also found to promote migration by acting as a chemotactic source resulting in cell migration towards the distal tip of the limb bud (Itoh et al., 1996; Takayama et al., 1996; Webb et al., 1997; Scaal et al., 1999).

Transcription factors are involved in delamination and migration, with Pax3 and Lbx1 being initially expressed in the lateral dermomyotome. Pax3 is necessary for the epithelial-mesenchymal transformation of the lateral dermomyotome (Daston et al., 1996). In *Pax3* null mice, which are also called *Splotch* mice, *c-met* expression is either significantly reduced or absent in the lateral dermomyotome. This suggests that *Pax3* is upstream of *c-met*, since the dermomyotome is disorganized and the limb myogenic cells do not migrate (Epstein et al., 1996; Yang et al., 1996; Mennerich et al., 1998; Tremblay et al., 1998). Inactivation of *Lbx1* causes premoygenic cells to delaminate properly but have dysfunctional migration. The resulting phenotype exhibits minimal hindlimb musculature and the extensors (or dorsal muscles) are missing in the forelimb. These observations are similar to experiments where *Gab1* is inactivated, which is also involved in c-met signaling and is also believed to be a result of cells being unable to respond to limb migratory cues (Schäfer and Braun, 1999; Brohmann et al., 2000). Therefore, as these premyogenic cells migrate distally towards the limb bud, they begin to switch on the myogenic determination bHLH transcription factors, *MyoD* and *Myf5*, and form the dorsal and ventral muscle masses. The myogenic cells then terminally differentiate into slow

or fast fibers, which express the relevant form of slow or fast MyHC. The fast fibers are needed for movement with the slow fibers helping maintain posture (Francis-West et al., 2003).

Another major player in skeletal muscle formation is Sonic hedgehog (Shh), which is expressed in the notochord and floor plate of the neural tube and induces the formation of the sclerotome. Along with the Wnt proteins (1, 3a, 7a, 11), Shh also promotes myogenesis by activating expression of *Myf5* in the epaxial myotome through its interaction with Wnt1. *Wnt1* is expressed in the dorsal neural tube and the dorsal ectoderm. *Wnt7a* is expressed in the dorsal ectoderm and found to activate the expression of *MyoD* in hypaxial myotome (Schmidt et al., 2000; Petropoulos and Skerjanc, 2002).

The bone morphogenic proteins (BMPs) are expressed in the lateral plate mesoderm and limb bud ectoderm. *Noggin*, an antagonist for BMP, is expressed in the dorsomedial lip of dermomyotome. BMPs and Noggin regulate *Pax3* in proliferating muscle precursor cells (MPCs), and *Pax3* is critical for the downstream activation for Myogenic Regulatory Factors (MRFs) (Amthor et al., 1998). The levels of BMPs within the somite, in the presence or absence of its antagonists, controls the expression of *Pax3* and can either promote embryonic muscle growth by expanding Pax3-expressing muscle precursor cells or restricting development by inducing apoptosis (Amthor et al., 2002). In the limb, *BMP2* and *BMP7* expression is increased by Shh. This causes a stimulation of muscle growth and a delay in muscle differentiation by allowing these BMPs to inhibit activation of *MyoD* and *Myf5* by Pax3 (Tajbakhsh et al., 1997). The BMP signals originating from the lateral plate delay the activation of myogenic bHLH gene expression (Pourquié et al., 1996). In the presence of Noggin, BMPs are sequestered, *Pax3* expression is repressed, and *MyoD* expression is dramatically expanded, inducing formation of a lateral myotome (Duprez et al., 1998; Reshef et al., 1998; Krüger et al., 2001). Conversely,

another BMP antagonist, follistatin, promotes *Pax3* expression, which transiently delays muscle differentiation and exerts a proliferative signal during muscle development (Amthor et al., 2002).

Myogenic Regulatory Factors (MRFs)

At the molecular level, MRFs mediate the process of myogenic determination and muscle-specific gene expression enabling multipotent, mesodermal cells to give rise to mononucleated myoblasts, withdraw from the cell cycle and differentiate into multinucleated muscle fibers, which are the framework of whole muscle (Cooper and Konigsberg, 1961; Stockdale and Holtzer 1961; Stockdale et al., 1999). MRFs are a family of skeletal muscle specific bHLH transcription factors and contain the genes, *Myf5*, *MyoD*, *MRF4*, and *myogenin*. When these factors are overexpressed in non-myogenic cell lines, they cause them to differentiate into myogenic cells (Davis et al., 1987; Wright et al., 1989; Braun et al., 1989). *MyoD* and *Myf5* are expressed in proliferating, undifferentiated cells and when cells begin to terminally differentiate, *myogenin* expression is induced (Smith et al., 1993, 1994; Andres and Walsh, 1996). *MRF4* is expressed in both early stages of myogenesis, during muscle development, as well as in adult muscle tissue (Hinterberger et al., 1991).

Another family of genes, the MEF2 family augments the myogenic activities of MRFs. (Shen et al., 2003). MyoD and MEF2 interact to control myoblast specification, differentiation, and proliferation and MRF4 acts in embryonic cells to control myogenic determination (Kassar-Duchossoy et al., 2004). However, *myogenin* expression is required for terminal differentiation before birth. In addition, the expression of skeletal muscle *α-actin* is a marker of late differentiation following *myogenin* expression (Knapp et al., 2006).

Further mediation of MRF activities is achieved through their heterodimer formation with another family of bHLH proteins, the E proteins (Lassar et al., 1991; Shirakata et al., 1993). The

E proteins are class I, bHLH proteins. These class I proteins form heterodimers with the class II bHLH proteins such as MyoD. This heterodimer binds to specific DNA regions (E boxes) that contain the sequence CANNTG and activate transcription of muscle target genes (Yutzey and Konieczny, 1992). E47 is an alternate splice product of the E2A gene expressed early in myogenic differentiation (Sun and Baltimore, 1991; Quong et al., 1993).

Emerging data demonstrates that the intracellular signaling pathway, p38 mitogen-activated protein kinase (MAPK), participates in several stages of the myogenesis. The most documented role of p38 is its cooperation with transcription factors from the MyoD and MEF2 families in the activation of muscle specific genes. This interaction contributes to the temporal gene expression during differentiation (Cuenda and Cohen, 1999; Zetser et al., 1999). Withdrawal of myoblasts at the G_1 stage is necessary for differentiation to occur. This is partially mediated by p38 kinase and c-Jun N-terminal protein kinases (JNKs) signaling through its induction of *p21* expression in myoblasts (Puri et al., 2000; Mauro et al., 2002). The MyoD/E47 heterodimer is regulated by p38 MAPK and MRF4 is phosphorylated by p38 MAPK on Ser-31 and Ser-42 resulting in a reduced transcriptional activity. The modulation of MRF4 activity also results in selective silencing of muscle-specific genes in terminal differentiation (Suelves et al., 2004). During somite development in mice, p38 MAPK plays a crucial role in activating MEF2 transcription factors. In *Xenopus laevis*, p38 signaling is required for early expression of *Myf5* and for the expression of several muscle structural genes (Keren et al., 2005).

Negative myogenic regulators exist such as, Id1 and Twist. Id1 is a dominant negative HLH protein that prevents the interaction of the E proteins with MyoD or Myf5 by preferentially binding to the E proteins (Norton et al., 1998). Twist complexes with E proteins to repress

myogenic differentiation. Twist also can directly block transcriptional activity of MyoD and MEF2 by binding to E-box sequences (Hebrok et al., 1997; Puri and Sartorelli, 2000).

In order for muscle differentiation to occur, myoblast cell cycle arrest is critical. MyoD promotes cell cycle arrest by inducing *p21*, which is a cyclin-dependent kinase (cdk) inhibitor (Guo et al., 1995; Halevy et al., 1995). Induction of *p21* is subsequent to *myogenin* expression and it is believed that high levels of p21 are required for cells to remain in a post-mitotic state. MyoD also induces retinoblastoma (*Rb*), which is a negative regulator of G_1 progression (Martelli et al., 1994). There is an intricately regulated pattern of cell cycle proteins that are temporally expressed based on a variety of molecular interactions and further discussion of these events is found in a later section. Briefly, G_1 progression is controlled by Cdk-mediated phosphorylation of the Rb protein, which allows for the release and activation of E2F1 (Lundberg and Weinberg, 1998). E2F1 mediates S-phase progression by allowing for expression of genes required for DNA replication and mitosis (Ishida et al., 2001). Withdrawal from the cell cycle is mediated by p21, which directly inhibits Cdk complexes and interferes with S-phase progression by binding to and inhibiting the activity of proliferating cell nuclear antigen (PCNA), a subunit of the DNA polymerase (Dotto, 2000).

Satellite Cells

More than 40 years ago, Alexander Mauro first identified muscle progenitor cells in frog skeletal muscle, which were identified as satellite cells based on their observed location adjacent to mature muscle fibers as seen by electron microscopy (Katz, 1961; Mauro, 1961). These undifferentiated cells have a degree of plasticity, demonstrating properties of stem cells such as, yielding all of the specialized cell types from which they originate and having the ability to self-renew. In mature muscle, in response to injury, hypertrophy, routine maintenance or disease, satellite cells serve as a reserve of muscle stem cells that are activated from their quiescent or G_0

state to re-enter the cell cycle and provide myonuclei for growth or repair (Moss and Leblond, 1971). Self-renewal ability in satellite cells may occur by either a stochastic event or through asymmetric cell division, and this self-renewal is required for maintenance of their own population pool (Collins et al., 2005). Satellite cells are derived from a Pax3/Pax7 population of progenitor cells located in fetal muscle (Relaix et al., 2006).

In a quiescent state, satellite cells are characterized by a low nucleus/cytoplasm ratio, few organelles, and high amount of heterochromatin or condensed chromosomes, resulting in minimal metabolic activity or transcription (Schultz, 1976). Quiescent satellite cells can also be distinguished from activated cells because there are numerous morphological changes that occur upon activation. During activation, cytoplasmic extensions become apparent, along with an increase in cytoplasmic volume of the activated cell, the amount of heterochromatin decreases and organelles such as the Golgi, endoplasmic reticulum, ribosomes, and mitochondria start to appear (Schultz et al., 1978). Additionally, at the molecular level, *MyoD* expression is turned on, and a CD34 isoform switch is observed, along with co-expression of Pax7, M-cadherin, and Myf5. Cellular proliferation and division is indicated by expression of proliferation markers such as PCNA, and induction of *myogenin* expression designates cells undergoing myogenic differentiation (Fuchtbauer and Westphal, 1992; Grounds et al., 1992; Yablonka-Reuveni et al., 1994; and Zammit et al., 2004).

Satellite Cell Marker Proteins

In order to distinguish satellite cells from other mononucleated cells within skeletal muscle, protein markers have been identified which are specific to satellite cells alone or in combination with other markers that allow for the specific identification of a pure population of satellite cells. Some of the most studied are CD34, M-cadherin, Pax7, syndecan-3/4, VCAM1, and c-met (Rosen et al., 1992; Beauchamp et al., 2000; Seale et al., 2000; Cornelison et al.,

2001). Pax7 is currently the best marker for identifying quiescent satellite cells (Seale et al., 2000). Markers like CD34 are useful in identifying satellite cells on isolated myofibers, but are not specific or unique to satellite cells and a co-staining or a combination of markers is needed to definitively identify these cells (Beauchamp et al., 2000). Although, the truncated form of CD34 (Beauchamp et al., 2000) and the β isoform of myocyte nuclear factor (MNF) are specific to quiescence (Garry et al., 1997; Yang et al., 1997). There is also a level of heterogeneity that exists within the satellite cell population in terms of immunohistochemical (IHC) cell staining and *in vitro* clonal analyses (Schultz and Lipton, 1982; Baroffio et al., 1996; Molnar et al., 1996). For example, most satellite cells are positive for both CD34 and M-cadherin and most of these cells are also positive for Myf5 (Beauchamp et al., 2000), a subpopulation of ~20% are not positive for these markers. Interestingly, functional studies suggest that this subpopulation of cells serves as a reserve for satellite cell replenishment (Rantanen et al., 1995; Schultz, 1996).

c-Met

c-Met is a transmembrane tyrosine kinase receptor, and represents the activated HGF receptor, which is found in all quiescent satellite cells but not expressed in myofibers (Allen et al., 1995; Cornelison and Wold, 1997; Tatsumi et al., 1998). However, it is not restricted to satellite cells, and is detected in both resting and regenerating muscle. The *c-met* knockout mouse demonstrates that c-met is necessary for proper limb, diaphragm, and skeletal muscle formation. Following a crush injury, the mononucleated cells surrounding the necrotic fiber express the c-met marker. Additionally, cells negative for another stem cell marker, CD34, but positive for c-met (CD34-/c-met+) are still capable of giving rise to myotubes in culture (Beauchamp et al., 2000).

Syndecans 3 and 4

Syndecans 3 and 4 have overlapping expression patterns in skeletal muscle with c-met and are cell surface trans-membrane heparin sulfate proteoglycans. They function in FGF signaling and the expression of Syndecan 3 and 4 is consistent with that of satellite cell position (between basal lamina and sarcolemma) and proliferating MPCs (Cornelison et al., 2001). *Syndecan 3* and *4* knockouts have severe defects in satellite cell activation and muscle regeneration (Cornelison et al., 2004). Syndecans play a role in signaling through the ERK1/2 MAP kinase pathway. Primary mouse satellite cells require heparin sulfate for normal proliferation (Cornelison et al., 2001). Activation and initiation of myogenesis is delayed *in vitro* when syndecan signaling events are blocked (Cornelison et al., 2004).

M-cadherin

M-cadherin is a calcium-dependent adhesion molecule, which is used as a marker of quiescent satellite cells and activated myogenic precursors, but is not expressed in differentiated myotubes (Irintchev et al., 1994; Moore and Walsh, 1993; Beauchamp et al., 2000; LaFramboise et al., 2003). It is expressed in some, but not all quiescent satellite cells, demonstrating heterogeneity within satellite cell compartments (Cornelison and Wold, 1997). Those that do express M-cadherin also express CD34 (hematopoietic stem cell marker) (Beauchamp et al., 2000). Studies suggest that M-cad+/CD34+ satellite cells are also Myf5-positive (Beauchamp et al., 2000). Another observation is that the number of M-cadherin positive cells increases upon satellite cell activation (Cornelison and Wold, 1997).

CD34

CD34 is expressed in cells that express no cardiac, hematopoetic, or skeletal muscle mRNA transcripts, indicating a lack of lineage. This follows with the idea that only a sub-population of satellite cells expresses the CD34 marker and designates a sub-population of

satellite cells that are less committed to the myogenic lineage (Beauchamp et al., 2000). Cells that express *CD34*, don't express *Pax7* and *M-cadherin*, and both CD34 positive and negative progenitor cells can give rise to myotubes *in vitro*. One study examined CD34-positive versus CD34-negative primary myoblasts and found that CD34-positive primary myoblasts were more efficient in participating in regeneration but CD34-negative cells had a higher fusion index *in vitro* (Beauchamp et al., 2000).

Pax7

Pax7 is a member of the paired box family of transcription factors, and is localized to nuclei situated in discreet peripheral locations within resting adult skeletal muscle (Seale et al., 2000; Relaix et al., 2005). The number of cells positive for Pax7 is believed to correlate well with the expected number of satellite cells. This is because Pax7 expression co-localizes with myostatin, c-met, and m-cadherin in satellite cells resting between basal lamina (Seale et al., 2000; LaFramboise et al., 2003; McCroskery et al., 2003; Halevy et al., 2004). In addition, myogenic cells lines, which model quiescent, undifferentiated myoblasts, appear to be uniquely marked with high levels of Pax7. Furthermore, the expression seen in proliferating primary myoblasts is down-regulated upon myoblast differentiation. Although loss of Pax7 does not induce differentiation, indicating that other factors must be present or absent for myoblasts to commence terminal differentiation (Seale et al., 2000; Olguin and Olwin, 2004).

Pax7 inhibits myogenic conversion induced by MyoD. It is believed that Pax7 is indirectly interfering with its function or competes for proteins necessary for MyoD-dependent transcription since the presence of Pax7 cannot inhibit the effects of MyoD-E47 heterodimers (Olguin and Olwin, 2004). Yet, overexpression of Pax7 in satellite cells does induce cell cycle exit, prevention of BrdU incorporation, a decrease in *MyoD* expression, and prevention of *myogenin* induction (Olguin and Olwin, 2004). In addition to its impact on MyoD, it is believed

that myoblasts expressing the Pax7+/MyoD- phenotype may return to quiescence to replenish satellite cell pools while myoblasts that acquire MyoD proliferate and fuse to form myotubes (Zammitt et al., 2004; Relaix et al., 2005). Alternatively, one study showed that Pax7 may have anti-apoptotic functions. Myoblasts transduced with dominant negative Pax7 lead to cell death (Relaix et al., 2006).

Myocyte nuclear factor (MNF)

MNF is a winged helix transcription factor, also identified as Foxk1, expressed in both cardiac and skeletal muscle, and in quiescent satellite cells. The number of MNF positive cells increases when muscle is induced to regenerate, and while detected in regenerating myotubes, *MNF* is down-regulated during late stages of regeneration (Garry et al., 1997). There are two isoforms of MNF, MNF-α and MNF-β, and both are detectable in skeletal muscle. Alpha is expressed in proliferating MPCs, while beta is limited to quiescent satellite cells and down-regulated upon activation (Yang et al., 2000). In quiescent satellite cells, high MNF-β expression allows for the formation of repressive complexes with mSin3 family members to repress targeted gene transcription (Garry et al., 2000; Yang et al., 2000). Upon satellite cells activation, isoform switching disrupts the repression by mSin3, allowing for the targeted genes to become active (Yang et al., 1997).

Notch

Notch signaling plays a role in cellular homoestasis and cell fate determination (Hing et al., 1994). In mammals, there are four genes, *Notch1*, *Notch2*, *Notch3*, and *Notch4* and five ligands, Dll-1, Dll-3, Dll-4, Jagged-1, and Jagged-2 (Chitnis et al., 1995; Lindsell et al., 1995; Li et al., 1998). The Notch receptors are transmembrane receptors and when bound by the extra-cellular ligands, Delta, Serrate, or Lag-2, this results in a cleavage of the intracellular domain of

the receptors in the cytoplasm. This cleavage product is the active form of Notch, NICD (Notch Intracellular Domain), which translocates to the nucleus where it binds to the family of transcriptional repressors, CSL (also known as RBP-Jκ, CBF-1, Suppressor of Hairless, and Lag-1). This interaction converts them into transcriptional activators (Nye et al., 1994; Ahmad et al., 1995).

The molecule Numb is an inhibitor of Notch but little is known about the regulation of Numb at the transcriptional level. Numb localizes to one pole during cell division in crescent-shaped patterns so that only one daughter cell receives Numb. This asymmetric expression is believed to play a role in determining which cell goes on to differentiate into a myofiber and which cell is going to replenish the satellite cell pool. Numb and Pax3 protein expression are mutually exclusive in satellite cells and a Pax3+/Numb- cell indicates a satellite cell that is less committed to a specific phenotype. The daughter cell retaining Numb expression is more committed to progressing along the myogenic lineage (Conboy and Rando, 2002).

Constitutively active Notch1 signaling causes up-regulation of *Pax3* and down-regulation of *Myf5*, *MyoD*, and *desmin* and a reduction in *Pax7* expression. In response to injury, activated Notch expression exhibits a highly localized expression at the site of injury, but not at more distal regions along the fiber. This suggests that it might play a role in targeting or recruiting specific factors to the injury site to promote regeneration (Conboy and Rando, 2002). The efficiency of tissue regeneration decreases in response to aging and it was proposed that this is due to age-related changes in satellite cell activity. Further analyses demonstrated insufficient up-regulation of Delta, resulting in diminished Notch activation in aged, regenerating muscle. When Notch was inhibited in young muscle, impaired regeneration was observed. Conversely,

when Notch was introduced into old muscle the regenerative potential was restored (Conboy et al., 2005).

To determine if systemic factors play a role in aged progenitor cells from specific tissues, parabiotic pairings between young and old mice were studied (Conboy et al., 2005). Interestingly, the exposure of satellite cells in old mice to young mouse serum resulted in enhanced expression of Delta, increased Notch activation, and enhanced proliferation *in vitro*, supporting the notion that there are systemic factors that change with age that impact progenitor cell activity (Conboy et al., 2005).

Growth Factor Effects on Satellite Cells

There are multiple growth factors that regulate satellite cell activation and growth. When myofibers are injured, the release of cytokines is stimulated, and growth factors, such as HGF (Allen et al., 1995; Tatsumi et al., 1998) and FGFs, cause an activation and expansion of satellite cells so that they will re-enter the cell cycle and rapidly proliferate, although activation of satellite cells has been found to be delayed over time in response to aging (Sheehan and Allen, 1999; Clarke et al., 1993; Johnson and Allen, 1995; Cornelison et al., 2001, 2004). Transforming growth factor beta (TGFβ) inhibits cell proliferation to some extent, although its more significant effect is in its inhibition of differentiation and fusion (Florini et al., 1986; Greene and Allen, 1991; Rao and Kohtz, 1995; Stewart et al., 2003; Allegra et al., 2004). MyoD, Myf5, and Pax7 are markers of the myoblasts at this stage (Davis et al., 1987; Wright et al., 1989; Braun et al., 1989; Rhodes and Konieczny, 1989; Seale et al., 2004). Factors such as TGFβ, HGF, and FGF inhibit myoblasts from undergoing differentiation and formation of myofibers (Gospodarowicz et la., 1976; Florini et al., 1986; Olsen et al., 1986; Massague et al., 1986; Miller et al., 2000).

Skeletal myoblasts serve as a good model to study intracellular signaling cascades as they undergo morphological changes characteristic of various cellular processes. Previous reports demonstrated the significance of the Raf kinase signaling axis (Bennett and Tonks, 1997; Coolican et al., 1997; Dorman and Johnson, 1999; Samuel et al., 1999; Dorman and Johnson, 2000; Winter and Arnold, 2000; DeChant et al., 2002). Elevated Raf levels are implicated in causing repression of myoblast differentiation and low-levels enhancing differentiation. These findings highlight the importance of time and duration of Raf signal transmission (DeChant et al., 2002). The molecular basis for inhibition of differentiation may involve direct modification of E-proteins and/or induction of TGFβ-like gene expression. Additionally, inhibition of myogenic differentiation has been demonstrated when cells were treated with various growth factors such as FGFs (1, 2, 4, 6, and 9), TGFβ, and high concentrations of serum (Allen and Boxhorn, 1989; Sheehan and Allen, 1999).

Insulin-like Growth Factor I (IGF-I)

IGF-I promotes cell recruitment to injured muscle by coordinating a regenerative response to muscle injury. Treatment of isolated satellite cells with IGF-I increases their proliferation rates in response to the JAK/STAT pathway. This growth factor induces both proliferation and subsequent differentiation of satellite cells via the type I receptor (Allen and Boxhorn, 1989; Adams and McCue, 1998; Kamanga-Sollo et al., 2004). Activation of myoblast proliferation is mediated through the MAPK pathway and induction of differentiation signals through the phosphatidylinositol-3-kinase (PI3K) pathway. In overloaded skeletal muscles, IGF-I peptide levels increase (Adams and Haddad, 1996). *In vivo* evidence demonstrates that when the tibialis anterior (TA) muscle of a rat is infused with IGF-I, there are measurable increases in muscle protein, muscle DNA content, and absolute weight of the treated muscle (Adams and McCue,

1998). Interestingly, *IGF-I* is up-regulated in regenerating muscles and in aged rats and application of IGF-I rescued approximately 46% of lost muscle mass and increased the proliferation potential of satellite cells from atrophied gastrocnemius muscle (Chakravarthy et al., 2000). In *mdx* mice (*dystrophin* null), high levels of muscle-specific IGF expression resulted in an increase of approximately 40% in muscle mass. A subsequent increase in force generation also was observed along with an elevation of signaling pathways associated with muscle regeneration and protection against apoptosis (Barton et al., 2002). IGF-I serves as a strong mitogen of satellite cells even in the presence of a strong growth inhibitor, TGFβ, yet the presence of TGFβ is able to inhibit IGF-I-mediated satellite cell differentiation (Allen and Boxhorn, 1989). Additionally, IGF-I increase the magnitude of the proliferative response elicited by FGF2 and stimulate differentiation when treated alone (Greene and Allen, 1991).

Fibroblast Growth Factors (FGFs)

In muscle tissue, there are several FGFs expressed and released in response to injury (Anderson et al., 1995). FGF2 and FGF6 are potent enhancers of muscle precursor cell (MPC) expansion and satellite cells by increasing proliferation and promoting muscle regeneration (Johnson and Allen, 1993; Lefaucheur and Sebille, 1995; Floss et al., 1997; Sheehan and Allen, 1999; Yablonka-Reuveni et al., 1999). FGF receptors (FGFR) 1, 2, 3, and 4 are expressed in proliferating rat satellite cells with FGFR1 and 4 being the most prominent (Sheehan and Allen, 1999). FGF2 is a heparin-binding growth factor that increases satellite cell proliferation and PCNA expression (Johnson and Allen, 1993). Treatment with FGF2 elicits a greater mitogenic response than IGF-I or TGFβl and is as potent as HGF in stimulating satellite cell proliferation (Sheehan and Allen, 1999). Combinations of FGF2 and HGF are additive with regard to

proliferation, and after injury, disrupted myofibers express FGF2, particularly in regions of hyper-contraction (Anderson et al., 1995).

FGF2 is present in newly formed myotubes and injection of FGF2 into the TA muscle of male *mdx* mice during the first round of spontaneous necrosis results in enhanced satellite cell proliferation. This is due to increasing the number of satellite cells that enter the cell cycle but inhibits the differentiation of satellite cells (Lefaucheur and Sebille, 1995). The *FGF6* knockout in certain genetic backgrounds exhibits a reduced regenerative capacity after crush injury and when they are interbred with *mdx* mice, a severe dystrophic phenotype is observed. *FGF6* is up-regulated in response to skeletal muscle injuries and helps completely restore experimentally damaged skeletal muscle (Floss et al., 1997). Higher levels of FGF2 and FGFR1 are expressed in the pectoralis major of turkey. Faster proliferating satellite cells from pectoralis major of turkey express higher levels of FGF2 and FGFR1 by comparison to slower proliferating cells. They also show a greater mitogenic response to FGF2, suggesting that FGFs may play a role in proliferative rates of muscle cells (McFarland et al., 2003).

Hepatocyte Growth Factor (HGF)

HGF, also called Scatter Factor (SF), is a critical activator of satellite cells found initially in crushed muscle extract (CME) (Tatsumi and Allen, 2004; Tatsumi et al., 2002). Direct injection of HGF into muscle results in activation of quiescent satellite cells, even in the absence of trauma, and when an anti-HGF antibody is incubated with CME, the activation capacity of the CME is abolished (Tatsumi et al., 1998; Tatsumi et al., 2002). This growth factor binds to the c-Met receptor and signals through the PI3K pathway to promote cell survival and MAPK pathways to stimulate the mitogenic effect (Allen et al., 1995). Placenta, liver, and muscle all express HGF (Brand-Saberi et al., 1996). In addition, HGF is present in basal lamina of skeletal muscle fibers, which provides a reservoir of HGF within skeletal muscle (Tatsumi and Allen,

2004). The *HGF* (-/-) mouse is embryonic lethal, and without HGF signaling, skeletal muscle cells cannot migrate from the somite during embryogenesis (Schmidt et al., 1995; Bladt et al. 2002).

Transforming Growth Factor Beta (TGFβ) Superfamily

The TGFβ superfamily is involved in cellular proliferation, differentiation, migration, and apoptosis. TGFβ is one of the most potent negative regulators of proliferation and differentiation of satellite cells (Florini et al., 1986; Greene and Allen, 1991; Rao and Koht, 1995; Stewart et al., 2003; Allegra et al., 2004). Inhibition of myoblast fusion is dose-dependent and reversible (Florini et al., 1986; Stewart et al., 2003).

The TGFβ superfamily can be divided into three groups: the TGFβs, the activins/inhibins, and the bone morphogenic proteins (BMPs). All three groups of growth factors signal through serine/threonine kinase receptors (Massagué et al., 1994). In the presence of growth factors, ligands bind to a Type II receptor dimer located on the plasma membrane, which causes auto-phosphorylation of the Type II dimer, recruitment of a Type I receptor dimer, and subsequent phosphorylation of this dimer (Wrana et al., 1992; Attisano et al., 1993; Ebner et al., 1993; Wieser et al., 1993; Wrana et al., 1994). This phosphorylation event recruits the receptor-regulated Smads or R-Smads, which then undergo phosphorylation (Aoki et al., 2001). The R-smads form a complex with the common-partner Smads (Co-Smads), or Smad4. The R-Smad/Co-Smad complex translocates to the nucleus and binds to the DNA altering transcription of target genes (Derynck et al., 1996; Liu et al., 1996; Meersseman et al. 1997; Nakao et al., 1997). Additional co-activators and co-repressors lend regulation to the system (Wotton et al., 1999).

Specificity of TGFβ signaling is mediated by the different types of Type II and Type I receptors present in the target cell and ligand affinities for these receptors (Wrana et al., 1992; Attisano et al., 1993; Ebner et al., 1993; Wieser et al., 1993; Wrana et al., 1994). Additionally, R-Smads (Smads 1, 2, 3, 5, and 8) are ligand-specific. Smads are cytoplasmic when inactive and transfer to the nucleus upon phosphorylation (Derynck et al., 1996; Liu et al., 1996; Meersseman et al. 1997; Nakao et al., 1997). I-Smads or inhibitory Smads (Smad6 and Smad7) bind to the receptor and prevent phosphorylation and signaling activities (Imamura et al., 1997; Nakao et al., 1997; Horiki et al., 2004). Negative regulation of TGFβ signaling is achieved by follistatin, which binds activin and prevents receptor docking (Nakamura et al., 1990). BMPs (2, 4, and 7) form a trimeric complex between ligand, receptor, and follistatin to inhibit BMP actions (Iemura et al., 1998). Table 1-1 summarizes the receptor and R-Smad specificity for TGFβ superfamily members (ten Dijke et al., 1994a; ten Dijke et al., 1994b; Koening et al., 1994; Macías-Silva et al., 1998; Yamashita et al., 1995; Rosenzweig et al., 1995; Liu et al., 1995; Nohno et al, 1995).

Myostatin

Myostatin (MSTN), also called growth and differentiation factor 8 (GDF8) is a member of the TGFβ superfamily and is responsible for maintaining muscle size. Mouse devoid of MSTN possesses a larger muscle mass characterized by hypertrophic fibers. *MSTN* is expressed in multiple tissues with the greatest mRNA levels found in muscle (Grobet al., 1997; Kambadur et al., 1997; McPherron and Lee, 1997). MSTN circulates in the blood in an inactive form until the pro-domain is cleaved away by a furin enzyme (McPherron et al., 1997; Lee and McPherron, 2001; Zimmers et al., 2002). Treatment with MSTN inhibits proliferation of muscle precursor cells. Fluorescence Activated Cell Sorting (FACS) analysis determined that MSTN prevents progression of myoblasts from G_1 to S-phase transition of the cell cycle. Subsequently,

upregulation of *p21* and a decrease of Cdk2 protein and activity is observed, which results in an accumulation of hypophorylated Rb and arrest in G_1 (Thomas et al., 2000). This same cell cycle arrest is observed in MSTN treated satellite cells. MSTN is thought to be required for maintaining satellite cells in their quiescent state, until inhibited by a stimulus such as injury (McCroskery et al., 2003). In MSTN-deficient satellite cells, a higher number of satellite cells are activated compared to wildtype counterparts and addition of MSTN to myofiber explant cultures inhibits satellite cell activation (McCroskery et al. 2003).

In the *MSTN* knockouts, hypertrophy and hyperplasia are observed (McPherron et al., 1997). These animals exhibit an increased number of satellite cells versus wild type counterparts, and the satellite cells present in the knockout animals have an increased proliferation rate (McCroskery et al., 2003). Gene ablation also results in enlarged hearts of the mutant animals. Myostatin signals through the Activin Receptor II and transgenic animals carrying a dominant negative receptor have a three-fold increase in muscle mass compared with wild type animals (Lee and McPherron, 2001).

Bone Morphogenic Proteins (BMPs)

BMPs are the largest group of family members in the TGFβ superfamily (Reddi and Huggins, 1972; Wozney et al., 1988). As the name implies, BMPs induce bone or cartilage formation ectopically and initiate osteoblast differentiation (Urist, 1965; Gitelman et al., 1995). BMPs are 30-38kDa homodimers that are synthesized as prepropeptides of approximately 400-525 amino acids. BMPs inhibit the myogenic differentiation of C2C12 cells, and convert their differentiation pathway into that of osteoblast lineage cells (Katagiri e al., 1994). Additionally, BMPs play a role in somite development by abrogating premature initiation of myogenesis in the presomitic mesoderm (Pourquie et al., 1996). A BMP inhibitory signal is

believed to prevent the premature expression of *MyoD* before somites are formed (Linker et al., 2003). In pre-myogenic cells, BMP2, 4, and 7 have dose-dependent effects with low concentrations maintaining a Pax3-expressing proliferative population and delaying differentiation. Conversely, high concentrations of these BMPs prevent muscle development (Amthor et al., 1998). *In vivo*, BMP2 expression in skeletal-derived cells prevents myogenic differentiation and promotes osteogenic differentiation (Musgrave et al. 2001).

BMPs mediate non-osteogenic processes. During the developmental and differentiation processes of the embryo, BMPs regulate epithelial-mesenchymal interactions, cell fate specification, dorsoventral patterning, apoptosis, and the secretion of extracellular matrix components (Vainio et al., 1993; Amthor et al., 1998; Weaver et al., 1999; Angerer et al., 2000; Higuchi et al., 2002; Tiso et al., 2002). More specifically, in vertebrates, BMP2, 4, and 7 are found to direct the development of neural crest cells into their ultimate phenotypes (Wilson and Hemmati-Brivanlou, 1995; Miya et al., 1997).

In terms of signaling, there are Type I and Type II receptors specific for BMPs and Smads1, 5, and 8 are the downstream molecules phosphorylated by the ligand-receptor complexes (see Table 1-1). At the transcriptional level, BMPs regulate target genes such as, *Runx2*, *osteopontin*, *osteonectin*, and *osteocalcin* specific for osteogenesis (Ahrens et al., 1993). Smad6 is an I-Smad. It functions by binding to type I BMP receptors thus preventing the activation of BMP Smads 1/5/8 (Imamura et al., 1997). Smad6 overexpression in chondrocytes results in delayed differentiation and maturation (Horiki et al., 2004).

Noggin and other cystine knot-containing BMP antagonists associate with BMPs to block their signaling. *Noggin* overexpression in osteoblasts, results in osteoporosis in mice (Devlin et al., 2003; Wu et al., 2003). Noggin is a secreted peptide, which is expressed in condensing

cartilage and immature chondrocytes. Ablation of *Noggin* is embryonic lethal at 18.5 days post coitum (dpc) and results in severe hyperplasia of the cartilage with multiple joint fusions, severe defects in somitogenesis, and multiple skeletal defects (Brunet et al., 1998; McMahon et al., 1998). Noggin is found to have different affinities for different BMP family members making its regulation of BMP-mediated processes more complex. For example, in the *Noggin* null transgenic, different bones have varying responses to the de-repression of BMP signaling (Zimmerman et al., 1996; Chang and Hemmati-Brivanlou, 1999). Depending on location and/or embryonic origin of the bones, one observes inhibition, delay or acceleration of ossification processes (Tylzanowski et al., 2006).

Another BMP negative regulator is *Tob*, which suppresses the activity of receptor-regulated Smads (1/5/8) (Bradbury et al., 1991; Fletcher et al. 1991; Yoshida et al., 2000). *Tob* null mice exhibit enhanced BMP signaling and increased bone formation (Yoshida et al., 2000). Negative BMP regulation also is achieved by mechanisms that target elements of BMP signaling for degradation. The Hect domain E3 ubiquitin ligase, Smurf1, targets Smad1 and 5 for degradation, in addition to interacting with and mediating the degradation of bone-specific transcription factors such as Runx2 (Zhao et al., 2003). Smurf1 can also target type I BMP receptors for degradation by interacting with Smad6. They form a complex that can be exported from the nucleus where it interacts with the BMP receptors to promote degradation (Murakami et al., 2003). Furthermore, when *Smurf1* is overexpressed in osteoblasts, postnatal bone formation is inhibited (Zhao et al., 2004).

While BMPs are capable of redirecting muscle mesenchyme cells to differentiate into bone tissue and stimulate bone formation *in vivo* (Urist, 1965), previous reports have demonstrated that mutations of BMP family members results in disruption of skeletal development (Kingsley

et al., 1992; Storm and Kingsley, 1999). Moreover, BMPs have opposing activities that are concentration-dependent. In the presence of low BMP levels, myogenic precursor cells are maintained in a proliferative state in developing limb bud, while high BMP levels induce cell death. Thus, BMPs can both stimulate and restrict muscle growth suggesting that a concentration gradient of BMPs is needed for the correct determination and maintenance of the myogenic program (Amthor et al., 1998; Amthor et al., 2002). While BMPs signal through the Smads, a multitude of different transcription factors are recruited, accounting for the diversity of functional BMP responses. One set of genes involved in skeletal development are the Hox family of transcription factors. In analyses of gain- or loss-of-function and naturally occurring mutations of Hox genes, BMPs play a central role in embryonic skeletal patterning (Manley and Capecchi, 1997; Yueh et al., 1998). Furthermore, several of the *Hox* genes interact with Smads and there is evidence that Hox gene expression may be regulated by BMPs further suggesting that BMPs are a critical mediator of skeletal myogenesis (Ladher et al., 1996; Tang et al. 1998; Liu et al., 2004).

BMP6, a member of the BMP subfamily, was originally isolated from phage plaques of a λg10-based cDNA library derived from 8.5-dpc murine embryos. The murine library was hybridized with a ^{32}P-labeled partial *Xenopus laevis* Vg-1 cDNA under low-stringency conditions (Derynck et al., 1988). Northern blot analyses of murine tissues illustrated that *BMP6* was present in muscle (Lyons et al., 1989). The human and bovine homologs were isolated from bone and designated *BMP6* (Celeste et al., 1990). Expression predominates in mature chondrocytes during endochondral ossification and BMP6 treatment stimulates chondrogenic and osteogenic phenotypes *in vitro* and induction of cartilage and bone formation *in vivo* (Gitelman et al., 1994; Gitelman et al., 1995; Yamaguchi et al., 1996). Adult muscles express

BMP6 (Lyons et al., 1989) and BMP6 expression increases in Raf-arrested myoblasts (Wang et al., 2004). In keratinocytes, treatment with BMP6 significantly decreases DNA synthesis (D'Souza et al., 2001) and triggers differentiation programs (Tennenbaum et al., 1996). In these differentiated keratinocyte cultures, E2F5 protein levels are significantly increased (D'Souza et al., 2001). Treatment of keratinocytes with TGFβ1 causes reversible cell cycle arrest without activating the differentiation program (Pierce et al., 1998; Dicker et al., 2000).

BMP Function *in vivo*: Lessons Learned from Knockouts

A compilation of the various BMPs and receptors targeted knockouts and their resulting phenotypes are presented in Table 1-2. *BMP5* null mice are called short ear mice, because these animals have reduced ear size in comparison to wild type animals, in addition to exhibiting reduced vertebral processes, and a reduced number of ribs and sesamoid bones (Green, 1968; Kingsley et al., 1992; King et al., 1994). Mice with a homozygous *BMP7* deletion are found to die at birth due to renal failure because of hypoplastic/dysplastic kidneys (Dudley et al., 1995; Luo et al., 1995; Wawersik et al., 1999). *BMP6* null mice are viable and fertile and exhibit no major defects, except for a delay in ossification of the developing sternum (Solloway et al., 1998). It is believed that *BMP2* may be functionally compensating for *BMP6* ablation since *BMP2* and *BMP6* are required for some overlapping or redundant functions (Solloway et al., 1998).

Utilization of BMPs in a clinical setting was demonstrated in various therapeutic interventions such as, bone defects, non-union fractures, spinal fusion, osteoporosis, and root canal surgery. Multiple studies utilizing BMP2 demonstrate its ability to promote healing of severe long bone defects in rats, rabbits, dogs, sheep and non-human primates (Murakami et al., 2002). Adenoviral administration of BMP2 mixed with a bioresorbable polymer and

mesenchymal stems cells was able to repair bone defects (Chang et al., 2003). RhBMP2 administered systemically increased mesenchymal stem cell activity and reversed age-related bone loss due to ovariectomies in different mouse models (Turgeman et al., 2002) demonstrating a potential application of BMPs in osteoporosis treatment. Interestingly, rhBMP2 has been used as a complete bone graft substitute in spinal fusion surgery and in some cases BMP2 has been more efficacious in promoting successful bone fusion as compared to autogenous bone grafts. BMP2 was used in other fusion applications such as intervertebral and lumbar posterolaterial fusions (Sandhu, 2003). BMPs can be used in dental applications since BMP2 induces bone formation around dental implants used in periodontal reconstruction. It has been suggested that BMPs could serve as an alternative to root canal surgeries (Schwartz et al., 1998; Cochran et al., 1999).

Current BMP6 Studies

Recently, BMP6 was able to induce matrix synthesis and induce differentiation of bovine ligaments fibroblasts providing another potential source of chondrocytes for tissue repair (Bobacz et al., 2006). Mesenchymal stem cells (MSCs) treated with BMP6 in combination with parathyroid hormone (PTH) and vitamin D(3) increased osteocalcin production. Osteocalcin is used as a marker for bone formation and in these MSCs, BMP6 enhanced calcium formation (Sammons et al., 2004). TGFβ and BMPs may act in an antagonistic manner towards each other. TGFβ inhibits chondrocyte maturation relatively early in differentiation by down-regulating *bmp6*, *ihh* (Indian hedgehog), and *colX* (collagen type X), which are genes specifically expressed by hypertrophic chondrocytes (Vortkamp et al., 1996; Ferguson et al., 2004).

BMPs are implicated in the development of cancers of the GI tract, breast, and prostate (Howe et al., 2001; Pouliot and Labrie, 2002; Brubaker et al., 2004). Currently, much focus is

being placed on TGFβ superfamily members as targets for cancer therapy due to their ability to suppress tumor progression by inhibiting growth of neoplastic tissues. BMP6 in the early stages of carcinogenesis inhibits benign and malignant skin tumor formation (Wach et al., 2001). BMP6 exerts an anti-proliferative and pro-apoptotic effects in multiple myeloma (Kawamura et al., 2000; Hjertner et al., 2001; Kersten et al., 2005).

Ras/Raf in Skeletal Muscle

A key regulator of signal transduction pathways controlling cell proliferation, differentiation, and oncogenesis is Ras, a monomeric guanine nucleotide-binding protein with intrinsic GTPase activity (Marshall, 1996). Ras is a molecular switch that cycle between a GTP-bound active and a GDP-bound inactive state (McCormick, 1993). Oncogenic H-Ras inhibits the differentiation of muscle cells independent of their continued proliferation (Olson et al., 1987). Ras inhibits myogenesis by disrupting MRF function, resulting in the inhibition of differentiation of muscle cells independent of proliferation (Ramocki et al., 1998). Ras proteins are localized at the cytoplasmic face of the plasma membrane and activated by a large number of extracellular stimuli, such as growth factors and hormones (McCormick, 1993; Bar-Sagi, 2001). A family of Ras effector molecules specifically binds to and is activated by Ras-GTP, which then carries out the downstream functions of activated Ras (Marshall, 1996). Some effectors of Ras are the Ral GTPase signaling pathway involved in the G_1 to S progression (Ramocki et al., 1998) and the PI3K/AKT pathway involved in cell survival (Murphy et al., 2002). All of the major Ras effectors have been tested for a role in the Ras-mediated inhibition of skeletal myogenesis but none of them are able to duplicate the effects of oncogenic Ras (Ramocki et al., 1997; Weyman et al., 1997).

The Raf Family

The Raf family consists of three serine/threonine specific kinases and are the best characterized effectors of Ras. Members include A-Raf, B-Raf, and c-Raf, also called Raf-1 (Marshall, 1996). There are three conserved regions (CR1-3) within Rafs consisting of two N-terminal regulatory domains (CR1 and CR2) and a C-terminal catalytic kinase domain (CR3). Rafs lead to the activation of the extracellular signal-related kinase (ERK) pathway, which mediates cellular proliferation and differentiation (Marshall, 1996).

Inactive Raf is cytosolic and translocates to the plasma membrane following activation by Ras (Chong and Guan, 2003). The activation of Raf is important in mediating growth factor gene expression and a major function of Raf protein kinase is to phosphorylate MEK1 and MEK2. Subsequently, ERK1 and ERK2 are phosphorylated on tyrosine and threonine residues. These activated ERKs then either phosphorylate numerous cytoplasmic targets or migrate to the nucleus to activate transcription factors such as c-fos and Elk1 (Huang et al, 1993). The Raf/MEK/ERK signaling cascade is required for cell cycle progression and overexpression causes cell transformation (Kolch et al., 1991; Cowley et al., 1994; Mansour et al., 1994). The sustained activation of this pathway is implicated in differentiation with prolonged ERK activated leading to differentiation of PC12 cells (Qui and Green, 1992). Raf-activated MEK-ERK cascades are likely participants in apoptosis because potent ERK activation can protect cells from apoptosis (Le Gall et al., 2000).

Signaling through the Raf/MEK/ERK controls several aspects of myogenesis. Overexpression of a constitutively active Raf and MEK results in reduced muscle gene transcription and these myoblasts are unable to form myocytes (Dorman and Johnson, 1999; Dorman and Johnson, 2000; Samuel et al., 1999; Winter and Arnold, 2000). Down-regulation of ERK activity by overexpression of MAPK phosphatase I (MKP-I), or through the use of

synthetic inhibitors, upregulates muscle specific gene transcription (Bennett and Tonks, 1997; Dorman and Johnson, 1999). By contrast, other reports have found no effect of ERK activity on muscle gene expression, myoblast fusion, and myoblast differentiation (Weyman et al., 1997; Jones et al., 2001). Myoblasts expressing low levels of Raf differentiate more efficiently than control cells again suggesting that ERK1/2 promotes myogenesis (Wang et al., 2004). In differentiating C2 myoblasts, ERK1/2 activity increases MyoD expression and transcriptional activity (Gredinger et al., 1998). Activated ERK1 is associated with myoblast proliferation and activated ERK2 is associated with myoblast differentiation (Sarbassov et al., 1997). ERK1 and ERK2 are both ubiquitously expressed in most tissues and similar in structure. *ERK1* null mice are viable, fertile, and of normal size (Nekrasora et al., 2005), whereas, *ERK2* null mice are embryonic lethal (Yao et al., 2003).

E2F Family

The major function of the E2F factors is cell cycle progression, in addition to playing roles in metabolic activities such as proliferation, differentiation, and apoptosis (Fujita et al., 2002). Genes expressed at the G_1/S transition contain E2F-binding sites within their promoters (DeGregori et al., 1995). E2F members are conserved throughout evolution from invertebrates to mammals and there are currently nine family members, which demonstrate tissue-specific activities (Dynlacht et al., 1994; D'Souza et al., 2001). Two major groups, activators and repressors, comprise the E2F family. E2F1, E2F2, and E2F3a are involved in positive cell cycle control and S-phase entry of quiescent cells. These members preferentially bind pRb with expression peaking in late G_1 and association with E2F-regulated promoters during the G_1-S transition. Ectopic expression of these members results in S-phase induction in serum-starved cells (DeGregori et al., 1997; Lukas et al., 1996; Leone et al., 1998; Humbert et al., 2000). E2F3b, E2F4 and E2F5 are classified as repressor proteins, due to their negative control of cell

cycle progression. E2F4 and E2F5 predominately bind p107 and p130 pocket proteins (DeGregori et al., 1997; Leone et al., 2000). E2F6, E2F7, and E2F8 also are classified as repressors but through a mechanism independent of pocket protein interaction because they lack the Rb-binding sequence at the C-terminus (Campanero et al., 2000). Members of the E2F family demonstrate differential mRNA expression throughout the cell cycle. For example, E2F1 and E2F2 mRNA increase in late G_1, and peak at the G_1/S transition, although E2F2 mRNA expresses at a lower level than E2F1. During quiescence, E2F3 and E2F5 mRNA are expressed, with E2F1 barely present. In early-to-mid G_1, E2F3 and E2F5 mRNA levels rise (Sardet et al., 1995; Pierce et al., 1998).

Members of the E2F family are grouped based on their homologous DNA binding domain (DBD). All of the E2Fs, except E2F7 and 8, and both DP proteins have a conserved DBD and a dimerization domain. E2F4 and E2F5 demonstrate a 72% amino acid identity to each other and a 35% amino acid identity to E2F1-3 (Vaishnav et al., 1998). E2F4 and E2F5 make up a subclass of the E2F family since their N-terminus lacks the cyclin A-binding domain found in the other members. The repressive E2Fs contain the Rb-binding sequence and have nuclear export sequences instead of nuclear localization sequences in the N-terminus (Helin et al., 1993; Sardet et al., 1995).

E2F Signaling Mechanism and Involvement of Pocket Proteins

The E2F proteins signal via the formation of active heterodimer complexes with DP proteins (Helin et al., 1993). E2Fs can dimerize with either DP1 or DP2, except for E2F7 which binds DNA in a DP-independent manner (Ormondroyd et al., 1995). These E2F complexes promote gene expression required for G_1 progression and DNA replication (Chen et al., 2004).

The mammalian Rb family of proteins (pRb, p107, and p130) is also referred to as the pocket proteins. The pocket protein motif allows for interactions with cellular proteins that

possess a LXCXE peptide motif (Lee et al., 1998). The LXCXE motif is present in E2Fs, the D-type cyclins (Gill et al., 1998), and HDACs. The pocket domain is separated into two functionally conserved regions, the A and B pockets. The spacer region is specific to each pocket protein and contains binding sites for cyclin/CDK complexes in p107 and p130 but not in pRb. p130 and p107 contain approximately 50% amino-acid identity and 30-35% identity to pRb (Ewen et al., 1991; Hannon et al., 1993). Overexpression of cDNAs coding for pocket proteins induces growth arrest at G_1 (Classon et al., 2000).

Rb was the first tumor suppressor gene cloned and loss of Rb function serves as a hallmark of oncogenic progression. pRb is a major G_1 checkpoint and inhibits S-phase entry. pRb promotes terminal differentiation, cell cycle exit and tissue specific gene expression (Dunaief et al., 1994; DeCaprio et al., 1989; Dick et al., 2000).

Cell Cycle Repression

Pocket protein dephosphorylation occurs from anaphase to G_1 or in response to growth inhibitory signals (Ludlow et al., 1993). Phosphorylation events can also lead to permanent inactivation of the pocket protein and possibly target it for degradation (Ma et al., 2003). The type of functional effect that pocket proteins have on a cell is dependent on what type of co-accessory proteins interact with the target gene (Stevaux et al., 2005). To repress gene transcription needed for the G_1 to S-phase transition, the pRb binds directly to the transactivation domain of E2F. pRb also recruits chromatin remodeling factors such as histone deacetylase I (HDAC1), SUV39H1, hBRM, and BRG1. These factors act on the surrounding nucleosome structure to remodel it, and promote histone acetylation/deacetylation and methylation events (Shao and Robbins, 1995). HDAC1 is recruited to E2F complexes by pRb to function in repressing *cyclin E* gene expression (Magnaghi-Jaulin et al., 1998). SUV38H1 is a methyltransferase that methylates K9 of histone H3 and cooperates with pRb in the repression of

E2F-responding promoters (Rea et al., 2000). hBRM and BRG1 are the mammalian homologs of SNF2/SWI2, which are yeast chromatin remodeling complexes and they associate with pRb (Strober et al., 1996; Kang et al., 2004).

E2Fs and Pocket Proteins in Skeletal Muscle

pRb is required for muscle differentiation and for transcription of myogenic bHLH factors. Functional Rb is required for the activity of MyoD-mediated transcriptional activation of myogenic genes (Gu et al., 1993). In models of inactive Rb, myoblast differentiation was inhibited *in vitro* and terminally differentiated myotube nuclei were able to reenter the cell cycle. In the absence of *pRb*, skeletal muscle cells exhibited ectopic DNA synthesis and/or apoptosis, and *pRb* null mice exhibit severe defects in skeletal muscle (Schneider et al., 1994; Novitch et al, 1996; Zacksenhaus et al., 1996; Novitch et al., 1999; de Bruin et al., 2003; Wu et al., 2003).

Interestingly, E2Fs in complex with p130 accumulate in cells such as, myoblasts, and melanocytes undergoing terminal differentiation (Shin et al., 1995). In rat L6 myoblasts, p107 is normally involved in regulation of E2F proteins during cell cycle progression. Exponentially growing L6 myoblasts demonstrate complexes of E2F and bound p107 throughout the cell cycle. During the differentiation of L6 cells, p107 levels are reduced and p130 levels are greatly increased, suggesting that p130 is a differentiation-specific regulator of E2F activity (Kiess et al., 1995).

In summary, skeletal myogenesis is regulated by a multitude of growth factors and signaling events. Since most studies have focused on the role of BMPs in embryonic development, little is known about its role in postnatal muscle. E2Fs are critical mediators of cell cycle progression. Satellite cells are requisite for growth and repair of skeletal muscle. It is not well understood how satellite cells are activated to enter the cell cycle nor how they exit the cell cycle to return to quiescence. E2F5 appears to have some role in Raf-arrested myoblasts and

BMP6 expression is high in these Raf-arrested cells. Therefore, these data will further assess the role of BMP6 and E2F5 in regulation of skeletal myogenesis and/or satellite cell biology.

Table 1-1. Receptor and R-Smad specificity for TGFβ superfamily members

Ligand	Type II Receptor	Type I Receptor	R-Smad
Activin	Act-R-II/IIB	ALK4	Smad2,3
TGFβ	TGFβRII	ALK5	Smad2,3
		ALK1	Smad1,5,8
BMP	BMPRII	ALK3	Smad1,5,8
	ActRII/IIB	ALK6	Smad1,5,8
		ALK2	Smad1,5,8

Table 1-2. TGFβ superfamily transgenic knockouts*

Genes	Phenotypes	References
Ligands		
Bmp2	Delayed primitive streak, small allantois, lack of amnion, heart defects, decreased number of PGCs.	Zhang and Bradley, 1996; Ying and Zhao, 2001
Bmp3	Increase bone density	Bahamonde and Lyons, 2001; Daluiski et al., 2001
Bmp4	Lack of allantois and PGCs, posterior truncation, heart defects, and lack of optic vesicle; heterozygotes-cystic kidney, craniofacial malformations, microphthalmia,	Winnier et al., 1995; Dunn et al., 1997; Furuta and Hogan, 1998; Lawson et al., 1999; Ying et al., 2000
Bmp5	Short ear phenotype including defects in skeleton, lung, and kidney	Green, 1968; Kingsley et al., 1992; King et al., 1994
Bmp6	Delayed sternum ossification	Solloway et al., 1998
Bmp7	Skeletal defects, kidney agenesis, eye defects	Dudley et al., 1995; Luo et al., 1995; Wawersik et al., 1999
Bmp8a	Defects in spermatogenesis and epididymis	Zhao et al., 1998
Bmp8b	Defects in PGC formation, testis cord formation, and spermatogenesis	Zhao et al., 1996; Ying et al., 2000; Yao et al., 2002
Bmp11 (Gdf11)	Defects in A-P patterning of axial skeleton	McPherron et al., 1999
Bmp12 (Gdf7)	Hydrocephalic abnormalities growth defects in seminal vesicle	Lee et al., 1998; Settle et al., 2001
Bmp15	Subfertile due to defects in oogenesis	Yan et al., 2001
Gdf1	Defects in left/right asymmetry	Rankin et al., 2000
Gdf5	*Brachypodism* (shorterned skeleton in limbs and reduced number of digit bones)	Storm et al., 1994; Storm and Kingsley, 1996
Gdf8 (Myostatin)	Skeletal muscle hypertrophy	McPherron et al., 1997
Gdf9	Sterile due to defects in oogenesis	Dong et al., 1996
Nodal	No gastrulation (lack of primitive streak); anterior neural pattern defects; placenta defects (increased number of trophoblast giant cells)	Conlon et al., 1994; Varlet et al., 1997; Lowe et al., 2001; Ma et al., 2001; Zhou et al., 1993
Lefty1	Abnormal left-right axis (left isomerism)	Meno et al., 1998
Lefty2	Extended streak, excessive mesoderm, left isomerism	Meno et al., 1999
Activinβa	Lack whiskers and low incisors and cleft palate	Matzuk et al., 1995b
Activinβb	Defects in eyelid development and female reproduction	Vassalli et al., 1994
Inhibinα	Ovarian cancer	Matzuk et al., 1992
MIS	Pseudo-hermaphrodites (female reproductive tract present in males)	Behringer et al., 1994
Tgfβ1	Hyperactive immunity and defects in angiogenesis	Shull et al., 1992; Dickson et al., 1995

Table 1-2 continued.

Genes	Phenotypes	References
Tgfβ2	Perinatal lethality due to multiple defects in heart, lung, limb, spinal column, eye, inner ear, and urogenital system	Sanford et al., 1997
Tgfβ3	Cleft palate	Proetzel et al., 1995
Receptors		
Alk1	Defects in embryonic angiogenesis	Oh et al., 2000
Alk2 (ActrIA)	Defects in mesoderm formation as a result of defective visceral endoderm	Gu et al., 1999; Mishina et al., 1999
Alk3 (BmprIA)	Defects in epiblast proliferation and no mesoderm formation in null mutants, impaired cardiac and limb development in conditional mutants	Mishina et al., 1995; Ahn et al., 2001; Gaussin et al., 2002
Alk4 (ActrIB)	Defects in epiblast differentiation and lack of mesoderm formation	Gu et al., 1998
Alk6 (BmprIB)	Defects in seminal vesicle development, female reproduction, and limb skeletal formation	Baur et al., 2000; Yi et al., 2000; Yi et al., 2001
BmprII	Defects in gastrulation/lack of mesoderm	Beppu et al., 2000
TgfbrII	Defects in vasculogenesis and hematopoiesis	Oshima et al., 1996
ActrIIa	Deficiency in reproduction due to suppressed FSH and mild defects in skeletal development	Matzuk et al., 1995a
ActrIIb	Defects in axial patterning and left-right asymmetry (45% right isomerism)	Oh and Li, 1997

*Adapted from Zhao, G.H. 2003. Consequences of Knocking out BMP signaling in the mouse. Genesis 35:43-56. Table 1, pgs.45-46.

CHAPTER 2
MATERIALS AND METHODS

Myoblast Cell Culture

C2C12 skeletal muscle satellite cells (Blau et al., 1985) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1% v/v penicillin/streptomycin, and 0.1% v/v gentamycin. 23A2 skeletal muscle cells (Konieczny and Emerson, 1984) were maintained in Basal Medium Eagle (BME) supplemented with 15% FBS, 1% v/v penicillin/streptomycin, 0.1% v/v gentamycin reagent solution, 1% v/v L-glutamine. 23A2RafER^{DD} myoblasts were derived from the parental 23A2 myoblasts and stably express a tamoxifen-inducible chimeric Raf protein. The estrogen receptor fused to the Raf kinase domain is unstable in the absence of the estrogen analog 4-hydroxytamoxifen (4HT). Addition of 4HT binds to the estrogen receptor and allows for a dose-dependent increase in Raf protein expression and kinase activity that initiates downstream ERK1/2 activation (Wang et al., 2004). 23A2RafER^{DD} myoblasts were maintained in the same media as 23A2 cells with the addition of 10mM puromycin (Wang et al., 2004). All culture media, supplements, and sera were purchased from Invitrogen, Carlsbad, CA. For induction of stable Raf expression, cells were washed with phosphate buffered saline (PBS), treated with 10 μg/ml protamine sulfate (CalBioChem, San Diego, CA) in serum-free BME for 10 minutes. Cells were starved in serum free BME for one hour and treated with 1μM 4-hydroxytamoxifen (4HT; Sigma, St. Louis, MO) in 2% FBS BME. Cells for immunofluorescence were cultured on 35 mm glass-bottomed plates (World Precision Institute, Sarasota, FL) coated with 10% v/v BD Matrigel Matrix HC (BD Biosciences, San Jose, CA).

Growth Factor Treatment, and BrdU Pulsing and Fixation

C2C12 cells were seeded on gelatin-coated tissue culture plates at a concentration of $1x10^5$ cells/well in a 6-well cluster. Myoblasts were treated in the absence and presence of human recombinant BMP6 (R&D Systems, Minneapolis, MN) at final concentration of 25 ng/ml for 48 hours in 2% FBS DMEM. When necessary, cells were pulsed with 10 μM bromodeoxyuridine (BrdU) during the last thirty minutes of treatment and then fixed in 70% Ethanol (EtOH) at 4°C for 30 minutes.

Plasmids and Transfections

Semi-confluent myoblasts were transfected by calcium phosphate precipitation formation. In brief, each well received DNA precipitate containing 1 μg of luciferase reporter, 0.5 μg of activator plasmid, 0.5 μg of kinase, and 50 ng of pRL-tk, a *Renilla* luciferase plasmid used as a monitor of transfection efficiency. The luciferase reporter plasmids used in the study were a multimerized BMP response element (BRE-luc) (Korchynskyi and ten Dijke, 2002), the internal response element of the quail troponin I gene (TnI-luc) (Johnson et al., 1996), a multimerized TGFβ responsive element (3TP-luc) (Wrana et al., 1992), an Immunoglobulin E box reporter plasmid (μE5-Luc) (Johnson et al, 1996), and E2F-TA-luc (Clontech, Mountain View, CA). Activator plasmids used were CMV-E47 (Page et al., 2004), CMV-E2F5 (OriGene Technologies Inc., Rockville, MD), and pEM-MyoD (Page et al., 2004). Cells were maintained in growth media or differentiation-permissive medium supplemented with human recombinant BMP6, and TGFβ (R&D Systems, Minneapolis, MN) for 48 hours prior to lysis and measurement of luciferase and β-galactosidase or *Renilla* luciferase activities (Promega, Madison, WI). Final concentrations of BMP6 were 1, 10, 25, or 100 ng/ml. TGFβ1 was supplemented at 10 ng/ml.

BrdU Staining and BrdU Incorporation

Cells were fixed in 70% ethanol for 10 minutes at room-temperature, followed by incubation in 2N Hydrochloric acid (HCl) for 1 hour at 37°C. Cells were washed well with Phosphate Buffered Saline (PBS) and then incubated in blocking buffer (PBS containing 5% horse serum (HS), and 0.1% Triton X-100) for 1 hour. Antigen was detected by incubation with Biotynylated Anti-BrdU IgG (H+L) (BA-2000) at 1:100 (Vector Laboratories, Burlingame, CA) for 1 hour, followed by incubation with HRP-Streptavidin at 1:100 (Vector Laboratories, Burlingame, CA) for 1 hour. Antibodies were diluted in blocking buffer (PBS containing 2% Horse Serum, and 0.1% Triton X-100). Visualization of BrdU staining was accomplished by addition of one part 3,3'-Diaminobenzidine tetrahydrochloride (DAB) and five parts Nickel Chloride (NiCl) in the presence of H_2O_2. Percentages of BrdU incorporation were calculated by dividing the number of BrdU positive nuclei by the total number of nuclei. The averages of a minimum of six microscopic fields at 200X per treatment are shown.

Immunofluorescent E2F5 Staining

23A2RafER^{DD} cells were permeabilized for twenty minutes (1X PBS, 0.01% Triton X-100), incubated in blocking buffer for one hour, and then incubated overnight at 4°C with E2F5 antibody (sc-999) (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:50 in blocking buffer (1X PBS, and 2% Horse Serum). Next, cells were incubated with Alexafluor 488 conjugated anti-rabbit diluted 1:100 in blocking buffer (Molecular Probes, Carlsbad, CA) for 45 minutes at room temperature. Secondary antibody controls also were included to demonstrate the specificity of an antibody's expression patterns. Hoechst dye (1 μg/ml final concentration) was included as a nuclear stain. Representative photomicrographs at 630X under oil immersion were captured with a Nikon 1200DMX digital camera.

RNA Isolation and Nylon Arrays

Total RNA was isolated from C2C12 myoblasts and myofibers and 23A2 myofibers with the use of a Stratagene RNA Easy Kit. Equal amounts (3 ug) of pooled RNA were reverse transcribed with Superscript II (BD BioScience, San Jose, CA) in the presence of biotin-UTP. Biotinylated cDNAs were used to probe a RT^2 Gene Expression Assay, a nylon mini-arrays containing 96 genes coding for various members of the TGFβ superfamily, their receptors, signaling intermediates, and transcriptional regulators (SuperArray Bioscience Corporation, Frederick, MD). Array membranes were pre-hybridized with 100 μg/ml heat-denatured salmon sperm DNA in GEAhyb Hybridization Solution (SuperArray Bioscience Corporation, Frederick, MD) for 2 hours at 60°C. Biotinylated probes were added to arrays in GEAhyb solution to hybridize overnight (O/N) at 60°C. Following hybridization, the membranes were washed twice in 2X SSC (saline sodium citrate), 1% SDS (sodium dodecyl sulfate) and twice in 0.1X SSC, 0.5% SDS for 15 minutes each at 60°C. After washing, membranes were blocked with GEAblocking solution Q for 40 minutes at room temperature. Alkaline phosphatase-conjugated streptavidin (AP) was diluted 1:8,000 with Buffer F and incubated with the membrane for 10 minutes at room temperature. Membranes were washed four times with 1X Buffer F for 5 minutes each and then rinsed twice with Buffer G and visualized with CDP-Star chemiluminescent substrate for 2-5 minutes at room temperature (SuperArray Bioscience Corporation, Frederick, MD) and exposure to X-ray film (XAR-5, Kodak).

Western Blots

C2C12 myoblasts were differentiated in the presence or absence of 100 ng/ml BMP6. After 48 hours, the cells were lysed in 4X SDS-PAGE sample buffer (250 nM Tris pH 6.8, 8% SDS, 40% glycerol, 0.4% β-mercaptoethanol) and protein concentrations were measured (Bio-

Rad; Bradford, 1976). Equal amounts of protein were electrophorectically separated through denaturing gels and transferred to nitrocellulose membrane. The blots were incubated with 5% nonfat dry milk or 5% bovine serum albumin (BSA) in TBST (10mM Tris, pH 8.0, 150nM NaCl, 0.1% Tween 20) to block nonspecific binding sites. Primary antibodies were diluted in blocking buffer, and the blots were incubated overnight at 4°C with shaking. Antibodies and dilutions included the following: anti-myogenin (F5D ascites, Developmental Hybridoma Bank, University of Iowa, 1:5,000), anti-myosin heavy chain (MF20 hybridoma supernatant, Developmental Hybridoma Bank, University of Iowa, IA, 1:5), anti-troponin T (R & D Systems, Minneapolis, MN, 1:2,500), anti-desmin (D3, hybridoma supernatant, Developmental Hybridoma Bank, University of Iowa, IA, 1:10), anti-phospho-Smad1/Smad5/Smad8 (Cell Signaling Technology, Beverly, MA, 1:1,000), anti-p38 (1:5,000), and anti-phospho-p38 (1:5,000) (Cell Signaling Technology, Beverly, MA, 1:2,000). After three times of washing with TBST for 15 minutes each, the blots were reacted with the appropriate peroxidase conjugated secondary antibody diluted 1:5,000 in blocking buffer for 60 minutes at room temperature. Visualization of protein bands was accomplished by chemiluminescence (ECL, Amersham Biosciences, Piscataway, NJ) and exposure to X-ray film (XAR-5, Kodak).

Alkaline Phosphatase Staining

C2C12 myoblasts were treated for 0, 1, 24 or 48 hours with 100 ng/ml BMP6 or vehicle alone (control). Cultures were fixed with 4% paraformaldehyde and alkaline phosphatase (ALP) activity measured by reaction with 165 μg/ml 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and 330 μg/ml Nitro Blue Tetrazolium (NBT) color development substrates (Promega, Madison, WI) for 18 hours at 37 C. Representative photomicrographs at 100X were captured under bright field conditions with a 1200DMX digital camera (Nikon).

p38 Inhibition Assays

Myoblasts were treated with 10 μM SB202190 (Upstate Biotechnology, Charlottesville, VA), an inhibitor of p38 for 1, 24, and 48 hours. SB202190 is a cell permeable pyridinyl imidazole that potently inhibits p38α/SAPK2a and p38β/SAPK2b. This specific p38 inhibitor does not effect ERK activity or SAPK/JNK MAP kinases. After treatment, cells were fixed with 4% paraformaldehyde and assessed for ALP activity measured by reaction with NBT + BCIP or lysed and assayed for TnI-Luc activity.

Apoptosis Analysis

Myoblasts were treated with 25 μM staurosporine (Sigma, St. Louis, MO) for 3 hours. Cells were lysed in 4X SDS-PAGE sample buffer and equal amounts of protein were electrophorectically separated through denaturing gels and transferred to nitrocellulose membrane. The blots were incubated with 5% nonfat dry milk in TBST and primary antibodies were diluted in blocking buffer. The blots were incubated overnight at 4°C with shaking. Antibodies and dilutions included the following: anti-Poly (ADP-ribose) polymerase (PARP) (1:1000, Cell Signaling Technology, Inc., Danvers, MA) and anti-Bcl-2 (1:500, BD Biosciences, San Jose, CA). After three times washing for 15 minutes each with TBST, the blots were reacted with the appropriate peroxidase conjugated secondary antibody diluted 1:5,000 in blocking buffer for 60 minutes at room temperature. Visualization of protein bands was accomplished by chemiluminescence and exposure to X-ray film.

Statistics

All data presented represents at least three independent experiments with a minimum of two to three replicates per treatment group. All numerical data were compared to appropriate controls and each other as indicated for each experiment and analyzed following the General

Linear Models (GLM) Procedures of the Statistical Analysis System (SAS) (SAS, 1988). Differences between treatments were calculated using predicted differences between the least-squares means of treatment divided by standard deviation. Classes were designated as cells, treatment (trt), replicates (rep), and relative luciferase units (rlu). The statistical model included rlu equals treatment and replicates (rlu = trt rep) as main effects. Data was presented as Means ± SEM. Treatments were considered significantly different when $P \leq 0.05$.

CHAPTER 3
DIFFERENTIAL EXPRESSION OF TGFβ SUPERFAMILY MEMBERS DURING SKELETAL MYOGENESIS

Objective

Our previous work demonstrated that activated Raf, a key regulator of the MEK/ERK pathway inhibits myogenesis based on its overall signaling intensity (Ramocki et al., 1997; Dorman and Johnson, 1999; Page et al., 2004; Wang et al., 2004). In myoblasts exhibiting strong Raf/Extracellular-Signal Regulated Kinase (ERK) signaling, *TGFβ1*, *GDF8*, and *BMP6* are up-regulated, suggesting that these TGFβ family proteins may serve as autocrine inhibitors of differentiation (Wang et al., 2004). These proteins are members of the TGFβ superfamily, which consist of the BMP, activin, and TGFβ subfamilies. Skeletal myogenesis is intricately regulated by differential gene expression of these ligands. $TGF\beta_1$ and GDF8 are inhibitors of proliferation and differentiation (Florini et al., 1986; Greene and Allen, 1991; Rao and Kohtz, 1995; Stewart et al., 2003; Allegra et al., 2004). Ablation of *MSTN* in mouse and the naturally occurring mutations found in some Belgium Blue cattle result in greater muscle mass (Grobet al., 1997; Kambadur et al., 1997; McPherron and Lee, 1997). Much less is known about BMP effects on myogenesis. The objective of these experiments was to examine TGFβ signaling components during myogenesis in embryonic myoblasts and a postnatal satellite cell derivative.

Differential Transcriptional Activity in Myoblasts versus Myofibers

Autocrine activity during the transition from myoblasts to myofibers was examined in C2C12 cells transfected with a TGFβ response element promoter reporter (3TP-Lux) (Wrana et al., 1992), a BMP responsive reporter gene (BRE-Luc) (Korchynskyi and ten Dijke, 2002) or a muscle reporter gene (TnI-Luc). Myoblasts were maintained in differentiation-permissive medium for 48 hours prior to lysis, and luciferase activity measured. As expected, TnI-Luc

levels were barely detectable in myoblasts and increased approximately 12-fold during the conversion to myofibers (Figure 3-1). Evaluation of BMP and TGFβ-driven transcription varied in myogenic cells. In myoblasts, 3TP-Lux relative activity is decreased during differentiation. Conversely, BMP-directed transcriptional activity doubled in myofibers as compared to myoblasts (Figure 3-1). The different reporter levels observed in myoblasts and myofibers suggests that autocrine loops are present that may contribute to distinct stages of myogenesis.

Differentiation TGFβ Gene Expression in Myoblasts and Myofibers

Numerical changes in reporter activity suggested differential expression of TGFβ proteins during myogenesis. RT^2 Gene Expression Assays were used to quantify changes in relative TGFβ superfamily member gene expression profiles in myoblasts and myofibers. Total RNA was isolated from 23A2 (embryonic myoblasts) and C2C12 (adult satellite cells) myofibers and evaluated by ethidium bromide impregnated formaldehyde agarose gels. Distinct 18S and 28S bands demonstrate intact RNA (Figure 3-2A). Equal amounts of RNA were reverse transcribed in the presence of biotin-UTP (Figure 3-2B). The TGFβ miniarray was hybridized with biotynlated cDNA according to manufacturer's recommendations (SuperArray). Results demonstrate *Stat1*, *Noggin*, and *Runx1* transcripts are more abundant in 23A2 myofibers than C2C12 myofibers. By contrast, *Bmp3*, *Bmpr1a*, *Itgb5*, and *Igfbp3* expression is greater in C2C12 myofibers than 23A2 myofibers (Figure 3-3). Appendix A provides a complete list of genes and spotting locations.

Total RNA was isolated from proliferating C2C12 myoblasts or differentiated myofibers and relative gene expression was determined by nylon array. Results demonstrate that BMP ligands and receptors, TGFβ ligands, and Runxs are expressed differentially during the transition from myoblasts to myofibers. Runx1 mRNA levels are low in mononucleated myoblasts and

increase in multinucleated myofibers; Runx2 mRNA levels are high in myoblasts and low in myofibers and TGFβ3 is low in myoblasts and increases in myofibers (Figure 3-4).

The BMP ligands are inductive and inhibitory in myogenesis (Tzahor et al., 2003). Expression of *BMP1*, *2*, *3*, *4*, and *6* were detectable in both myoblasts and myofibers. Closer examination of these BMPs shows that BMP3, BMP4 and BMP6 mRNA levels are greater in mononucleated myoblasts than multinucleated myofibers. *BMP2* expression does not appear to differ between myoblasts and myofibers. BMP1 mRNA slightly increases from myoblast to myofibers (Figure 3-5). Relative amounts of *BMP1*, *3*, *4*, and *6* messages differ in myoblasts versus myofibers suggesting these genes may be involved in distinct stages of muscle formation.

BMP6 expression is greater in myoblasts than myofibers. Autocrine regulation of *BMP6* was examined in C2C12 myofibers. Results demonstrated that *BMP6* levels, and other BMP ligands, are not affected by ectopic BMP6 treatment (Figure 3-6). In summary, differential gene expression of TGFβ superfamily members is detected in different myoblast cell lines and during various stages of myogenesis. BMP6 mRNA is abundant in myoblasts but does not up-regulate it own expression.

Discussion

Assessment of autocrine activity during the transition from myoblast to myofiber by evaluation of BMP-responsive, TGFβ-responsive or muscle specific reporter elements demonstrated the presence of autocrine loops that may contribute to distinct stages of myogenesis. TnI-Luc activity drastically increased during the conversion of myoblasts to myofibers, which confirmed successful differentiation. TGFβ-sensitivity was high in myoblasts and low in myofibers, supporting the idea that a TGFβ-like protein is inhibiting differentiation. Conversely, BMP-sensitivity was low in myoblasts and high in myofibers. The different reporter

levels observed in myoblasts and myofibers further suggests that there are differential responses for TGFβ and BMPs at different stages of skeletal myogenesis or myoblasts versus myofibers.

23A2 cells are embryonic myoblasts that were derived from C3H10T1/2 embryonic fibroblasts treated with 5-Azacytidine (Konieczny and Emerson, 1984). C2C12 are adult satellite cells derived from murine limb muscle (Blau et al., 1985). While both of these immortalized cell lines exhibit myogenic properties, there are also marked differences between 23A2 and C2C12 cells. Comparison of embryonic myoblasts and adult satellite cells demonstrated detectable gene expression differences. Transcripts for *Stat1*, *Noggin*, and *Runx1* are greater in embryonic myoblasts than adult satellite cells. *Stat1* is involved in modulating anti-proliferative and growth arrest signals by inducing expression of cell cycle inhibitors, $p21^{WAF1/CIP}$ and pro-apoptotic signals (Durbin et al., 1996; Meraz et al., 1996). The *Stat1* knockout mouse demonstrates no overt developmental abnormalities although the mice have a significant increase in bone mineral density and bone mineral content (Xiao et al., 2004). Greater levels of *Stat1* in 23A2 myofibers may explain why ALP induction is greater in C2C12 than 23A2 myoblasts since it may act as a negative regulator of osteogenic activity.

Conversely, *Bmp3*, *Bmpr1a*, *Itgb5*, and *Igfbp3* transcripts are greater in adult satellite cells than embryonic myoblasts. *Bmp3* is the most abundantly expressed member of the BMP subfamily in both mononucleated myoblasts and multinucleated myofibers assessed by nylon array. BMP3 is also the most abundant BMP in adult bone and a major component of osteogenin, which does have osteogenic activity (Wozney and Rosen, 1993; Luyten et al., 1989). However, recombinant BMP3 is unable to induce an osteogenic response in multiple cell lines (Bahamonde and Lyons, 2001; Daluiski et al., 2001). Knockout models demonstrate that *Bmp3* is a negative determinant of bone density with null mice exhibiting twice as much trabecular

bone as wildtype counterparts. Additionally, BMP3 inhibits BMP2-responsiveness in osteoprogenitor cells and acts as an antagonist of osteogenic BMPs by activating the TGFβ/activin pathway, which would antagonize BMP signaling (Bahamonde and Lyons, 2001; Daluiski et al., 2001). Therefore, the abundant gene expression of BMP3 would be important for skeletal muscle formation because high levels of *Bmp3* play an essential role in modulating osteogenic BMPs. Specifically, *Bmp3* would help inhibit osteogenic conversion of myoblasts through competition for signaling components similar to both the TGFβ/activin and BMP pathways such as Smad4 (Heldin et al., 1997).

Gene expression profiles for myoblasts and myofibers demonstrated differential gene expression such as *Runx1* and *TGFβ3* are greater in myofibers than myoblasts and *Runx2* is greater in myoblasts than myofibers. Results suggest that these genes play a role in cell origin and regulate distinct stages of skeletal myogenesis. *Runx2* (or Cbfa1) is the master regulator for bone development and the *Runx2* knockout mouse demonstrates a complete lack of bone formation and chondrocyte hypertrophy in most of the skeleton (Shum and Nuckolls, 2002). Based on nylon array results, *Runx2* is greater in myoblasts than myofibers and this may be a function of the ability of myoblasts to undergo transdifferentiation in response to BMP6. Alternatively, myofibers and fibroblasts do not undergo transdifferentiation, which may be due to lower levels of *Runx2*. Nylon arrays demonstrated that there are differential gene expression profiles between 23A2 and C2C12 myofibers. Additionally, both lines were found to undergo transdifferentiation in response to ectopic BMP6 treatment, but to different magnitudes which also demonstrates differences in the molecular signaling of these immortalized lines.

In conclusion, these nylon arrays allow for a glimpse of differential gene expression of embryonic myoblasts and postnatal satellite cells, and different stages of skeletal myogenesis.

Verification of these results by Real-Time PCR analyses are needed with specific primers for each uniquely expressed gene. While previous literature demonstrates a role for these BMPs in embryonic development and somite patterning (Winnier et al., 1995; Zhang and Bradley, 1996; Dunn et al., 1997; Solloway et al., 1998; Daluiski et al., 2001; Ying and Zhao, 2001), the role of BMPs in adult myogenic cells has not been highly researched and this would determine which TGFβ superfamily members, specifically BMP genes, are important for molecular regulation of myogenic differentiation and fusion.

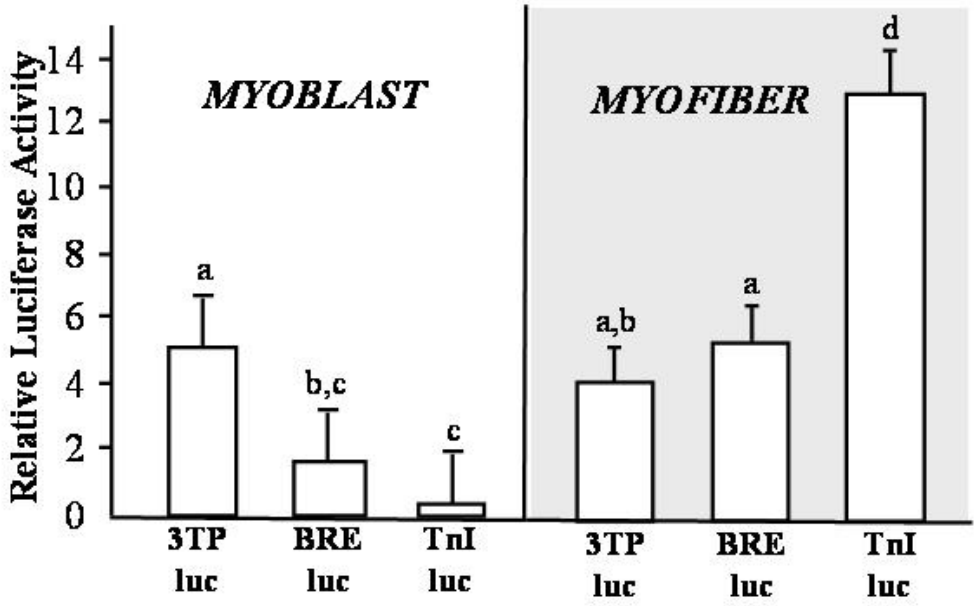


Figure 3-1. Differential transcriptional activity in myoblasts versus myofibers. C2C12 myoblasts (1 x 10^5) were transfected with 1 μg of 3TP-luc (TGFβ-specific), BRE-luc (BMP-responsive), or TnI-luc (Muscle-specific). Cells were maintained in growth medium (myoblasts) or differentiation-permissive medium (myofibers) for 48 hours. Cells were lysed, and luciferase and *Renilla* luciferase activities were measured. Means and SEM are from three independent experiments. Different letters indicates a significant difference, $P<0.05$.

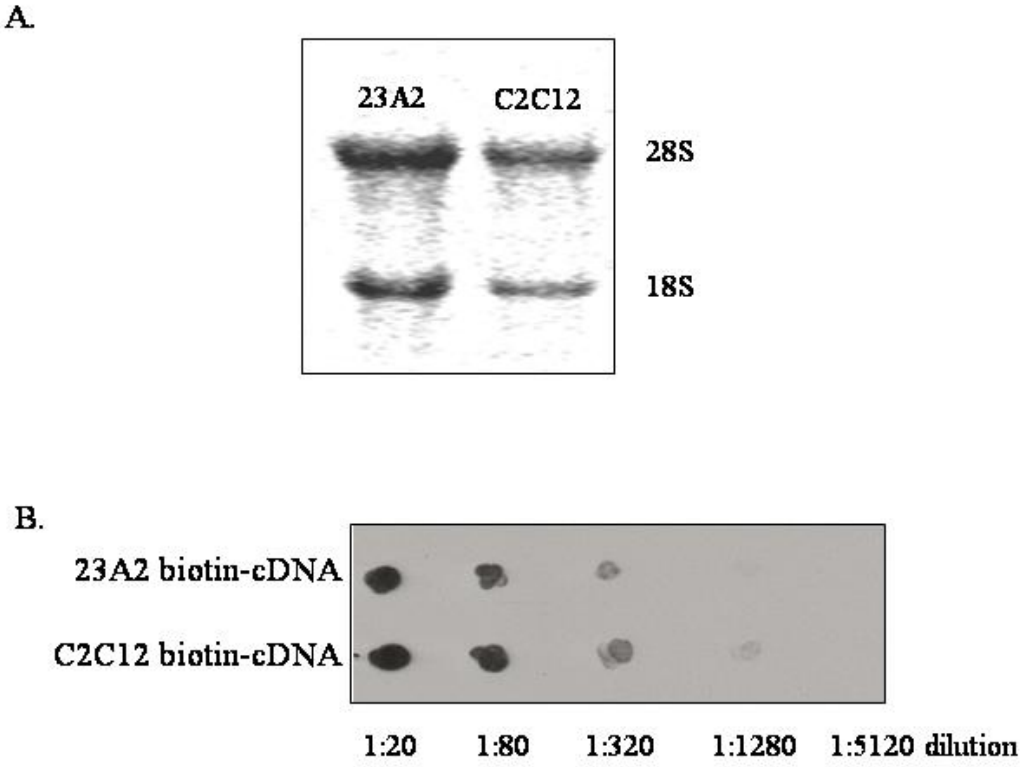


Figure 3-2. Intact RNA and cDNA probe synthesis. Total RNA was isolated from 23A2 and C2C12 myofibers. 15 μg was electrophoretically separated through formaldehyde agarose gels. RNA integrity was visualized with ethidium bromide, 18S and 28S ribosomal RNA are noted (A). One μg of RNA was reverse transcribed in the presence of biotin-UTP. Serial dilutions of biotin cDNA were spotted to nylon and incubated with streptavidin HRP. Chemiluminescence demonstrates biotin-UTP incorporation (B).

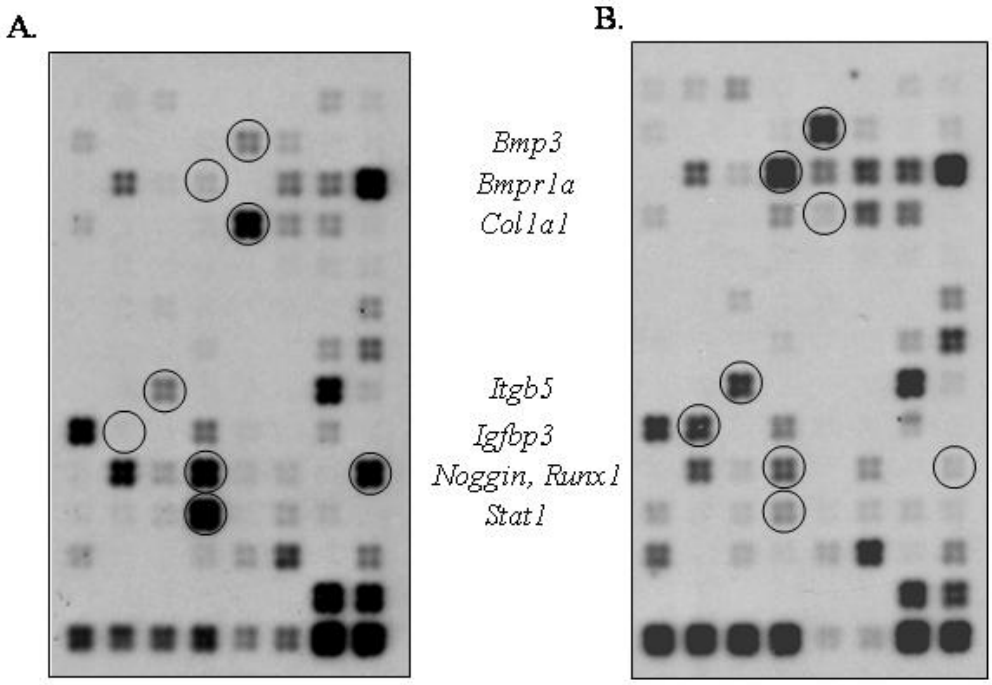


Figure 3-3. Gene expression profiles in embryonic and satellite cell myofibers. Total RNA was isolated from 23A2 embryonic myofibers (A) or C2C12 satellite cell myofibers (B) and equal amounts (3 μg) of RNA were reverse transcribed in the presence of biotin-UTP. Biotinylated cDNAs were used to probe a TGFβ/BMP Signaling Array (SuperArray). Following hybridization, the blots were incubated with avidin-peroxidase and visualized by chemiluminescence. Representative blots are shown. Significant gene expression differences are indicated in the column between the two arrays. Abbreviations: *Bmp3*, Bone Morphogenetic Protein 3, *Bmpr1a*, Bone Morphogenetic Protein Receptor 1a, *Col1a1*, Procollagen, type I, alpha 1, *Itgb5*, Integrin beta 5, *Igfbp3*, Insulin-like growth factor binding protein 3, *Runx1*, Runt related transcription factor 1, and *Stat1*, Signal transducer and activator of transcription 1.

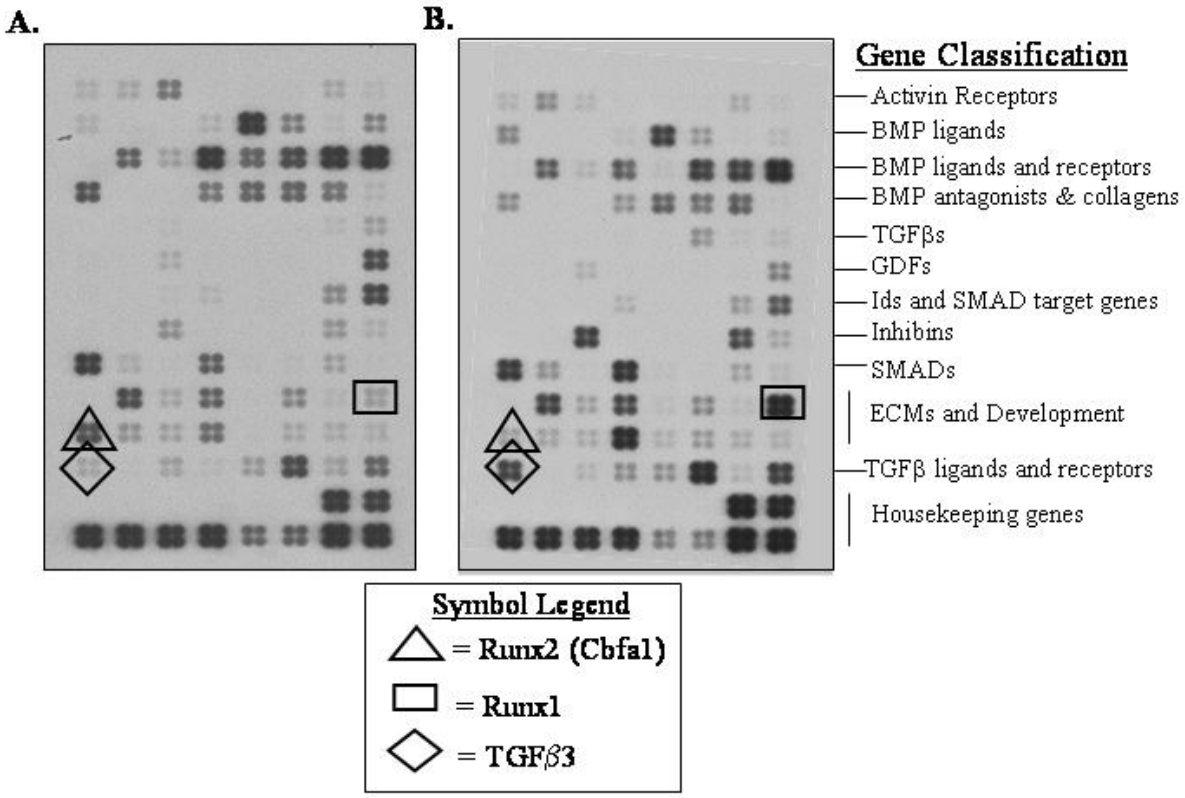


Figure 3-4. Differential gene expression in myoblasts and myofibers. Total RNA was isolated from C2C12 myoblasts (A) or myofibers (B) and equal amounts (3 μg) of RNA were reverse transcribed in the presence of biotin-UTP. Biotinylated cDNAs were used to probe a TGFβ/BMP Signaling Array (SuperArray). Following hybridization, the blots were incubated with avidin-peroxidase and visualized by chemiluminescence. Representative blots are shown. Gene Classification Groups are indicated on the right of the diagram. Abbreviations: BMP, Bone Morphogenic Protein, TGFβ, Transforming Growth Factor beta, GDFs, Growth and Differentiation Factors, Ids, Inhibitor of DNA binding, SMAD, MAD homolog, ECMs, Extracellular Matrix Molecules.

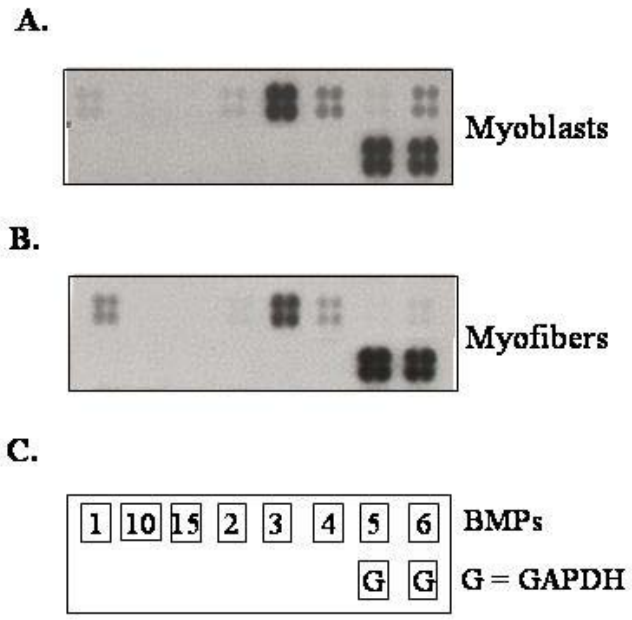


Figure 3-5. Relative BMP gene expression in myoblasts and myofibers. Total RNA was isolated from C2C12 myoblasts and myofibers and equal amounts (3 μg) of RNA were reverse transcribed in the presence of biotin-UTP. Biotynlated cDNAs were used to probe a TGFβ/BMP signaling array (SuperArray). Following hybridization, blots were incubated with avidin-peroxidase and visualized by chemiluminescence. Representative subsections of blots for BMP 1, 2, 3, 4, 5, 6, 10, and 15 are shown. (A) Myoblasts; (B) Myofibers; (C) Schematic location of genes.

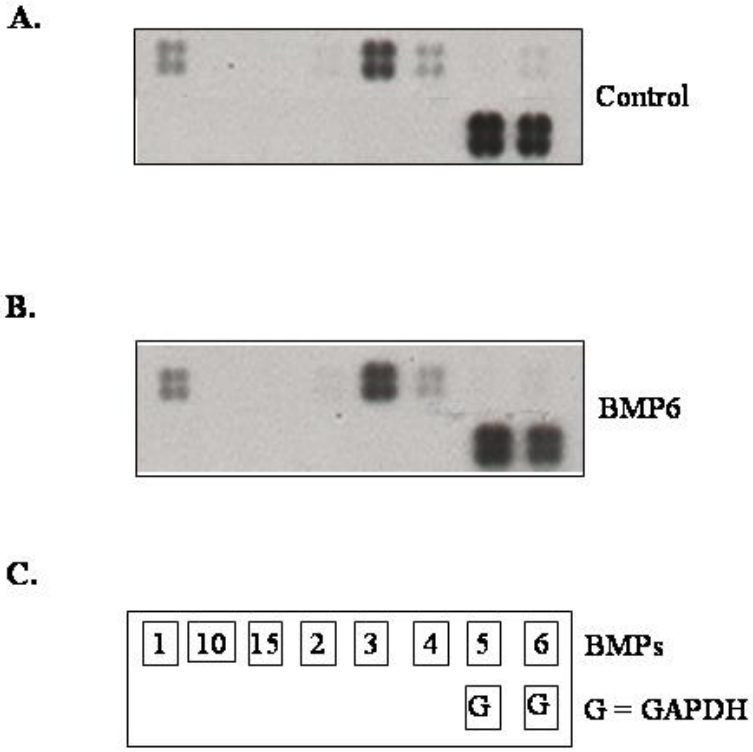


Figure 3-6. BMP6 does not undergo autocrine gene regulation. C2C12 myoblasts were differentiated for 48 hours in the absence (A) or presence (B) of 100 ng/ml BMP6. Total RNA was isolated and equal amounts (3μg) were reverse transcribed in the presence of biotin-UTP. Biotynlated cDNAs were used to probe a TGFβ/BMP signaling array (SuperArray). Following hybridization, blots were incubated with avidin-peroxidase and visualized by chemiluminescence. Schematic location of BMP genes (C).

CHAPTER 4
IMPACT OF BMP6 ON SKELETAL MYOGENESIS

Objective

In Raf-arrested myoblasts, removal of TGFβ_1 biological activity does not restore the myogenic program suggesting that another factor is mediating this inhibition (Wang et al., 2004). Ectopic GDF8 treatment does not inhibit 23A2 myoblast differentiation. BMP6 mRNA is present in myoblasts and absent in myofibers suggesting an inhibitory action during myogenesis (Derynck, 1989). The balance of proliferation, cell differentiation, and apoptosis mediates the pool of myoblasts available for skeletal myogenic maintenance. The objective of these experiments was to measure BMP6 effects on myoblast proliferation, differentiation, and apoptosis.

Inhibition of Skeletal Myogenic Differentiation by BMP6

To further examine the role of BMP6 in embryonic myoblasts and satellite cells, 23A2 myoblasts and C2C12 satellite cells were transiently transfected with TnI-Luc and treated with 0 or 100 ng/ml BMP6 for 48 hours in differentiation media prior to lysis and measurement of luciferase activity. Results show that treatment with 100 ng/ml BMP6 resulted in more than an 80-fold inhibition of muscle specific reporter (TnI-Luc) activity in both myogenic cell types (Figure 4-1, $P<0.05$). Due to the significant biochemical inhibition of differentiation by BMP6, assessment of morphological changes was performed. C2C12 myoblasts were differentiated in the presence of 100 ng/ml BMP6 for 48 hours. Subsequently, cells were fixed and immunostained for MyHC. Results show very few multinucleated fibers in response to BMP6 treatment (Figure 4-2A). Total cell lysates were isolated from a second set of C2C12 myofibers treated in an analogous manner. Equal amounts of protein (10μg) were analyzed by Western blot for contractile and regulatory protein expression. Inhibition of the muscle contractile protein

MyHC, in addition to, myogenin, and troponin T protein markers was observed in response to BMP6 treatment (Figure 4-2B). Thus, BMP6 inhibits the complete differentiation program.

Dose-Dependent Effects of Recombinant BMP6 on Myoblasts

BMP 2, *4*, and *7* have opposing activities that are concentration-dependent during embryonic muscle growth (Amthor et al., 1998; Amthor et al., 2002). Due to the high concentration of BMP6, a retrospective dose-response experiment was performed (1 ng/ml to 50 ng/ml BMP6). Biochemical differentiation was measured following transient transfection of TnI-Luc into C2C12 myoblasts. Cells were treated for 48 hours in differentiation media with increasing concentrations of BMP6. Analysis of luciferase reporter activity shows an approximate 20% decrease of TnI-Luc activity at 25 ng/ml ($P<0.05$) and an approximate 50% decrease at 50 ng/ml ($P<0.001$) (Figure 4-3A). Parallel plates were lysed for protein analyses. Equal amounts of total cell proteins were separated by SDS-PAGE and transferred to nitrocellulose. Blots were probed with anti-MyHC and anti-myogenin. Results demonstrate that MyHC protein expression begins to decrease at 50 ng/ml BMP6 (Figure 4-3B). Interestingly, myogenin protein expression begins to decrease at 10 ng/ml BMP6, further at 25 ng/ml and is completely absent in response to 50 ng/ml BMP6 (Figure 4-3B).

Induction of ALP Activity in Response to BMP6

BMPs induce bone or cartilage formation ectopically (Urist, 1965; Gitelman et al., 1995). BMP2 inhibits the myogenic differentiation of C2C12 cells, by converting their differentiation pathway into that of osteoblast lineage cells (Katagiri et al., 1994). To determine if the block to differentiation is associated with transdifferentiation, assessment of alkaline phosphatase (ALP) activity of myofibers treated with BMP6 was measured. C2C12 myoblasts were treated with vehicle only or increasing amounts of BMP6 in differentiation media for 48 hours. Cells were

fixed and histologically stained for ALP activity, a marker enzyme of osteogenic cells. As shown in Figure 4-4, as little as 5 ng/ml BMP6 was sufficient to induce ALP activity.

To determine if the effect of BMP6 is unique to the committed myoblast, C3H10T1/2 fibroblasts were treated with 0, 1, 10, and 100 ng/ml BMP6 in differentiation media for 48 hours. Cells were fixed and histologically stained for ALP activity. Results indicated that BMP6 does not cause ALP induction in C3H10T1/2 fibroblasts (Figure 4-5).

BMP6 Induces Rapid Transdifferentiation in Myoblasts

C2C12 myoblasts were treated with 100 ng/ml BMP6 for 24 and 48 hours. Subsequently, cells were fixed, and ALP activity was measured. Results show that ALP activity is observed as early as 24 hours and further increases at 48 hours (Figure 4-6). C2C12 cells treated with vehicle alone also demonstrated a slight induction of ALP at 24 and 48 hours indicating endogenous ALP activity.

BMP6 Does Not Alter Proliferation Rates of Myoblasts

Cell cycle withdrawal or inhibition of proliferation is required for differentiation and fusion of myofibers. To determine if the block to myogenic differentiation by BMP6 is due to altered cell proliferation, C2C12 myoblasts were treated with 25 ng/ml BMP6 for 48 hours. Cells were pulsed with BrdU during the final thirty minutes of the treatment interval, fixed with methanol, and immunostained for BrdU (Figure 4-7A). The numbers of nuclei were not different between control and BMP6 treated cells (Figure 4-7B, $P<0.05$). Therefore, exposure of myoblasts to BMP6 does not alter cellular proliferation.

BMP6 is not Anti-Apoptotic

Previous reports indicate that BMP2 and 4 promote cell survival in pluripotent mesenchymal cells by inhibiting TNF-mediated apoptosis (Chen et al., 2001). BMP6 was demonstrated to partially restore survivability in human mesenchymal stem cells (hMSCs)

induced by the BMP antagonist sclerostin (Sutherland et al., 2004). Bcl-2 is an anti-apoptotic protein expressed abundantly in satellite cells (Krajnak et al, 2006). The protective actions of BMP6 were examined in C2C12 myoblasts treated with staurosporine. C2C12 satellite cells were treated with increasing concentrations of BMP6 for 48 hours and Bcl-2 protein content measured by Western blot. Results demonstrate no changes in Bcl-2 protein concentration following BMP6 treatment (Figure 4-8A). Subsequently, myoblasts were treated for 3 hours with BMP6, 25 μM staurosporine or BMP6 and staurosporine. Cells were lysed and assayed for Poly (ADP-ribose) polymerase (PARP) protein expression by Western blot analysis. Results demonstrate that staurosporine stimulates PARP cleavage, a hallmark of apoptosis (Figure 4-8B). BMP6 does not prevent PARP cleavage in C2C12 myoblasts treated with staurosporine, thus precluding an anti-apoptotic function (Figure 4-8B).

In summary, BMP6 significantly inhibits the complete differentiation program as observed by biochemical suppression of muscle specific reporter activity, and morphological disruption of myofiber formation and muscle-specific protein expression. This inhibitory effect is dose dependent and results in rapid transdifferentiation of committed mesodermally-derived myoblasts to an osteogenic lineage. BMP6 does not inhibit differentiation by promotion of a proliferative state. Nor does BMP6 serve as an anti-apoptotic factor in myoblasts.

Discussion

BMP6 is greater in myoblasts than myofibers, and up-regulated in Raf-arrested myoblasts. Therefore, the function of BMP6 on proliferation, differentiation, and apoptosis in skeletal myogenesis was evaluated. Treatment of 23A2 and C2C12 myoblasts with 100 ng/ml BMP6 significantly inhibited muscle specific activity (TnI-luc). Muscle fiber fusion and muscle specific protein expression of myosin heavy chain (MyHC), myogenin and Troponin T are also

decreased in response to BMP6 treatment. Results suggest that BMP6 inhibits the complete differentiation program.

Other members of the TGFβ superfamily can also inhibit differentiation. One of the most notable examples is GDF8 or myostatin. *Myostatin* is mutated in double muscled cattle breeds, Belgium Blue and Piedmontese (Grobet et al., 1997; Kambadur et al., 1997; McPherron and Lee, 1997). It is predominately expressed in the muscle and negatively regulates myogenic proliferation. The *myostatin* knockout mouse also demonstrates two times larger muscle mass as compared to wild type counterparts (Thomas et al., 2000). Therefore, TGFβ superfamily members are critical mediators of skeletal myogenesis.

BMP6 is a morphogen, and morphogens are characterized by exhibiting different effects at different levels or concentrations. In pre-myogenic cells, BMP2, 4, and 7 have dose dependent effects with low concentrations maintaining a Pax3-expressing proliferative population and delaying differentiation. Conversely, high concentrations of these BMPs prevent muscle development (Amthor et al., 1998). In the presence of low BMP levels, myogenic precursor cells are maintained in a proliferative state in developing limb bud, while high BMP levels induce cell death. Thus, BMPs can both stimulate and restrict muscle growth (Amthor et al., 1998; Amthor et al., 2002). This suggests that a concentration gradient of BMPs is needed for the correct determination and maintenance of the myogenic program (Centrella et al., 1994; Alliston et al., 2001; Reddi, 1994). 23A2 myoblasts treated with increasing concentrations of BMP6 were measured for muscle specific reporter activity and MyHC and myogenin protein expression. Results demonstrated that muscle reporter activity is significantly decreased at both 25 ng/ml and 50 ng/ml. MyHC protein expression began to decrease at 50 ng/ml BMP6 and myogenin decreased at 10 ng/ml BMP6. This suggests that BMP6 exhibits dose dependent

effects on contractile and regulatory muscle proteins. Smad1 and Smad5 binding sites are found within the promoter of *myogenin*, which allow BMP6 to induce downstream Smad signaling. C2C12 cells transiently transfected with Smad1 and Smad5 were able to induced ALP activity and decrease myogenin/chloramphenical acetyltransferase (myogenin-CAT) activity. Although in NIH3T3 fibroblasts, Smad1 and Smad5 decreased myogenin-CAT but did not induced ALP activity, which demonstrates that Smad1 and Smad5 are involved in the BMP signaling that inhibits myogenic differentiation and induces transdifferentiation. Furthermore, the conversion of these two differentiation pathways is regulated independently at the transcriptional level (Yamamato et al., 1997). Additionally, high levels of BMP6 impact or repress stages of skeletal myogenesis prior to terminal differentiation.

Promotion of osteogenic differentiation by BMP2 expression in skeletal muscle-derived C2C12 cells (Musgrave et al. 2001) demonstrated that myoblasts can undergo transdifferentiation from a myogenic to osteogenic cellular lineage in response to BMP. ALP staining measures osteogenic activity and ectopic BMP6 treatment of C2C12 myoblasts demonstrated a dose dependent induction of ALP activity. Additionally, when C2C12 myoblasts were treated for 24 and 48 hours, induction of ALP activity in response to BMP6 treatment demonstrated a time dependent transdifferentiation of myofibers into an osteogenic lineage. The substantial induction in the numbers of ALP positive cells in C2C12 cultures maintained in low serum at 24 and 48 hours also suggests a basal level of osteogenic activity in C2C12 myofibers. These cells are mononucleates and not myofibers. Comparison of gene transcripts between myoblasts and myofibers by nylon array suggests that greater transcripts of *Runx1* could explain why these cells demonstrate a basal level of osteogenic activity. Furthermore, *BMP1*, *3*, and *4* transcripts are expressed in both untreated and BMP6 treated myoblasts, but only *BMP4*

displays osteogenic activity, which could contribute to endogenous ALP activity of C2C12 myoblasts. BMPs 2, 4, 6, 7, and 9 are characterized as osteogenic BMPs, with BMPs 2, 7, 6, and 9 displaying the highest osteogenic activity when applied ectopically *in vitro* and *in vivo* (Luu et al., 2007).

BMP1 is a metalloprotease that regulates deposition of fibrous extracellular matrix (ECM) in vertebrates and does not display osteogenic activity (Bond and Beynon, 1995). It provides procollagen C proteinase (PCP) activity to cleave the C propeptides of procollagens I-III to yield the major fibrous components of ECM (Kessler et al., 1996; Li et al., 1996; Suzuki et al., 1996; Scott et al., 1999). In bone, BMP1 co-purifies with TGFβ-like BMPs from osteogenic extracts of bone and is believed to coordinate the deposition of ECM with the activation of certain BMPs in early development and later in the development of bone and other tissues (Wozney et al., 1988; Scott et al., 2000). Furthermore, BMP3 would not contribute to endogenous ALP activity because as mentioned previously, recombinant BMP3 does not display osteogenic activity and acts as an antagonist of osteogenic BMPs (Bahamonde and Lyons, 2001; Daluiski et al., 2001).

Conversely, exposure of C3H10T1/2 fibroblasts to a BMP6 dose-response curve did not induce ALP activity at any dosage for 1, 10, and 100 ng/ml BMP6. The differential responses observed in C2C12 and C3H10T1/2 cells suggest that BMP6-mediated transdifferentiation is specific to lineage restricted cells. Similar results of a significant ALP induction in C2C12 cells by BMPs (2, 4, 6, 7, and 9) was reported by other groups. C2C12 cells demonstrated the most potent ALP induction (Ebisawa et al., 1999). Therefore, the origin of the cell line may determine if the cells will respond to BMPs and/or the overall magnitude of the ALP response. Yang et al., (2003) did demonstrate induction of ALP activity and stimulation of osteoblast marker genes by 300ng/ml of recombinant BMP6, whereas, our experiments never used a dosage higher than

100ng/ml BMP6. Another group demonstrated an induction of ALP activity in C3H10T1/2 fibroblasts in response to 100 ng/ml BMP2, suggesting that other BMP family members or higher dosages of BMP6 may have different impacts on fibroblasts. Additionally, BMP2-mediated ALP induction in C3H10T1/2 fibroblasts was increased in the presence of HGF (Imai et al., 2005). Therefore, the addition of HGF with BMP6 might result in a detectable ALP induction in C3H10T1/2 fibroblasts.

Comparison of BMP2 and BMP6 finds that both are expressed in skeletal muscle cells but are in different classes of the BMP subfamily (Lyon et al, 1989). BMP2 is a member of the DPP class along with BMP4, whereas, BMP6 is a member of the 60A class along with BMP5, 7, 8A, and 8B (Gitelman et al., 1997). Structurally, BMP2 contains 3 exons, two of which encode the precursor protein (Feng et al., 1994). The non-coding exon refers to an exon located on the 5' flanking region of the DNA that has been shown to serve as an alternate promoter, which suggests that BMP2 is regulated in both a developmental and tissue-specific manner (Gitelman et al., 1994). BMP6 is composed of 7 coding exons with the mature protein encoded by 3 full exons and a portion of the fourth exon and is also developmentally regulated. While BMP2 and BMP6 are members of the same subfamily, there is little similarity in the localization of the intron-exon structures further demonstrating differences at the structural level (Gitelman et al., 1994).

In vivo analyses demonstrates that *BMP6* null mice are viable and fertile and exhibit no major defects in known BMP6-expressing tissues, except for a delay in ossification restricted to the developing sternum (Solloway et al., 1998). It is believed that *BMP2* may be functionally compensating for *BMP6* ablation since *BMP2* and *BMP6* are required for some overlapping or redundant functions (Solloway et al., 1998). Based on nylon arrays, *BMP6* is greater in

myoblasts than myofibers, yet *BMP2* and *BMP4* transcripts don't appear to differ in myoblasts and myofibers. Conversely, when the muscles of athymic nude rats were injected with adenoviral vectors for BMP6 (AdBMP6), a rapid tissue calcification was observed. The induction of bone was produced through mechanisms similar to both intramembranous and endochondral ossification pathways and AdBMP6 was even more potent than the prototypical adenoviral vector AdBMP2 (Jane et al., 2002). These studies utilized titers of BMP6 that would not mimic physiological conditions but do demonstrate the powerful osteogenic activity of BMP6. *BMP2* null mice exhibit multiple developmental defects including a delayed primitive streak, small allantois, lack of amnion, heart defects and a decreased number of primordial germ cells (Zhang and Bradley, 1996; Yin and Zhao, 2001). Therefore, BMP2 and BMP6 appear to have different biological functions.

It has also been suggested that receptor oligomerization determines BMP2 signaling pathways. Nohe et al., (2002) demonstrated that binding of BMP2 to preformed receptor complexes activates the Smad pathway. Conversely, BMP2-induced recruitment of receptors activates a Smad-independent pathway, which results in the induction of ALP activity via p38 MAPK (Nohe et al., 2002). These different receptor complexes may also recruit different adaptor proteins such as, XIAP (Yamaguchi et al., 1999), BRAM-1 (Kurozumi et al., 1998), and FKBP12 (Wang et al., 1996). BMP2 treatment of C3H10T1/2 cells stimulates ERK1 and ERK2 during osteoblastic differentiation (Lou e al., 2000), and ERK activation can inhibit nuclear translocation of Smad1, which would block the Smad pathway (Kretzschmar et al., 1997). Our experiments demonstrate a strong Smad1/5/8 activation by BMP6 and only a slight p38 activation suggesting that BMP6 induces ALP activity through a different signaling mechanism than BMP2.

Characterization of BMP6 and BMP2 in C2C12 cells demonstrates that BMP6 strongly binds to activin receptor-like kinase (ALK)-2 or ActR-I (Ebisawa et al., 1999). ALK2 forms complexes with receptors like, BMPR-II or ActR-II. BMP6 can also weakly bind to ALK3, which also can bind BMP2 (Ebisawa et al., 1999) but BMP2 preferentially binds to BMPRIA and IB. C2C12 cells only express mRNA for BMPRIA (Akiyama et al., 1997). C3H10T1/2 fibroblasts express both BMPRIA and BMPRIB but BMPRIA expression levels are endogenously higher. Transfection experiments with BMPRIA and BMPRIB in C3H10T1/2 cells also demonstrate that the dominant role in BMP2 mediated osteogenic development was mediated by BMPRIA, with BMPRIB only partially influencing osteogenic development (Kaps et al., 2004). Additionally, type IB and IA BMP receptors appear to transmit different signals during the specification and differentiation of mesenchymal lineages (Kaps et al., 2004). Truncation and overexpression of BMP receptors, BMPRIA and BMPRIB, have demonstrated that overall receptor levels expressed in cells play a critical role in specification and differentiation of osteoblasts by BMP2 (Chen et al., 1998). Therefore, while BMP2 and BMP6 can interact with the same BMP receptor, they demonstrate the strongest affinity for different type I receptors. Therefore, differences in signaling could be based on like receptor oligomerization, cross-talk with other signaling pathways, competition for signaling components, interaction with additional proteins, and activation of different downstream transcriptional targets, which would result in different biological responses of BMP2 and BMP6.

Transdifferentiation of myogenic cells to an osteogenic linage results in induction of specific markers at the transcriptional level. Therefore, measurement of genes such as, *Runx1*, *Runx2*, *osteopontin*, *osteonectin*, and *osteocalcin* (Ahrens et al., 1993) would determine if the cells were exhibiting more "bone-like" characteristics and what genes are up-regulated in BMP6-

treated myoblasts. Interestingly, based on the nylon array assessment of myoblasts versus fibers, *Runx1* appeared to be greater in myofibers, yet *Runx2* appeared to be greater in myoblasts. *Runx2* is required for later stages of chondrocyte and osteoblast differentiation, while *Runx1* mediates early events of endochondral and intramembranous bone formation (Smith et al., 2004). These results would need to be confirmed by Real-Time PCR and may have implications in transcriptional regulation of the myoblast to myofiber transition and/or transdifferentiation of myoblasts to osteoblasts. Since myogenic precursor cells (MPCs) also have the ability to form skeletal muscle, bone, or cartilage, the differential expression of these transcription factors may demonstrate how these precursor cells determine their ultimate cell fate.

Inhibition of embryonic skeletal muscle differentiation through promotion of a proliferative state was monitored. Others have suggested that Raf inhibits skeletal myogenesis by keeping myoblasts in a proliferative state (Samuel et al., 1999). Since BMP6 expression is high in Raf-arrested myoblasts, it was proposed that BMP6 may also be promoting a proliferative state of cells. When myoblasts were treated with BMP6, the number of BrdU positive nuclei or cells in S-phase was not significantly different from untreated myoblasts. The percent BrdU incorporation was calculated by dividing the number of BrdU positive nuclei by the total number of cells and taking the average of six fields. There also was no significant difference in BMP6 treated percent BrdU incorporation versus control myoblasts. Therefore, exposure of myoblasts to BMP6 does not inhibit proliferation suggesting that BMP6-mediated inhibition of differentiation is not mediated through modulation of proliferative events. Conversely, another group did demonstrate an inhibition of proliferation by [^{3}H] thymidine incorporation assays in a dose-dependent manner (Ebisawa et al., 1999). Comparatively, this study did a dose response up to 1000 ng/ml BMP6 and only observed approximately a 20%

decrease at 300ng/ml, whereas our analyses assessed BrdU incorporation at 25 ng/ml, which is considered a more physiological dose.

Apoptosis is a mechanism of programmed cell death that promotes tissue turnover, embryonic development, and immunological defense mechanisms (Kerr et al., 1972; Adams, 2000; Siu et al., 2005). The intrinsic and extrinsic signaling pathways are the two principal pathways involved in apoptosis. The intrinsic route uses cell signaling pathways to alter mitochondrial function. Permeabilization of the outer mitochondrial membrane results in release of cytochrome c, thereby forming an apoptosome. This macromolecular complex activates caspase-9, mediated by apoptotic protease activign factor (APAF)-1 (Roy and Nicholson, 2000). Conversely, the extrinsic pathway sends 'death ligand' signals of the the Tumor Necrosis Factor (TNF) or Fas families through appropriate receptors that activate caspase-8 in conjunction with the adaptor molecule, Fas-associated death domain (FADD). At this point, intrinsic and extrinsic cascades converge to activate effector caspases (caspase-3 and -7), which cause the proteolytic degradation of cellular material (Roy and Nicholson, 2000).

BMP2, BMP4, and BMP6 promote cell survival in mesenchymal cells (Chen et al., 2001; Sutherland et al., 2004). Yet when myoblasts were treated with ectopic BMP6, Bcl-2 protein expression, an anti-apoptotic marker, did not change in response to increasing amounts of BMP6. Additionally, BMP6 did not prevent PARP cleavage in response to staurosporine, a common inducer of apoptosis. This suggests that BMP6 does not have an anti-apoptotic function in skeletal myoblasts. BMP6 was able to partially protect hMSC cells from sclerostin-induced apoptosis by decreasing caspase activity (Sutherland et al., 2004). Sclerostin is a BMP antagonist that binds to BMPs and blocks downstream signaling pathways. Furthermore, the protective effect of BMP6 observed was only a partial block of sclerostin-mediated apoptosis

suggesting that additional factors are involved (Winkler et al., 2003). Therefore, the protective effect of BMP6 observed in this scenario may be a result of additional signaling components not present in myoblasts or is due to an alternative apoptotic signaling cascade specific to Sclerostin or different from staurosporine-induced apoptosis.

In conclusion, BMP6 inhibits the complete differentiation program in myoblasts. BMP6 treatment results in a rapid transdifferentiation of myoblasts that is specific to a committed mesodermal derived myogenic cell. BMP6 does not appear to involve modulation of proliferation rates of myoblasts. Nor does BMP6 appear to demonstrate a survival role in myoblasts.

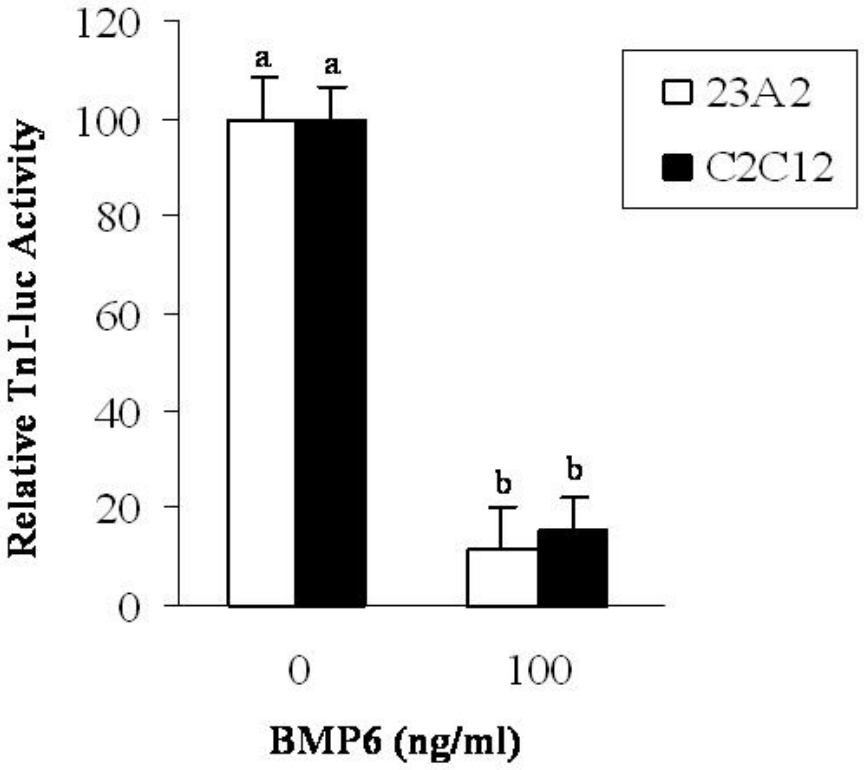


Figure 4-1. Biochemical inhibition of skeletal myogenesis by BMP6. Myoblasts were transiently transfected with 2 μg of TnI-Luc reporter construct and 50 ng pRL-tk (Renilla) and treated 0 or 100ng/ml BMP6 in differentiation media for 48 hours. Cells were lysed and assessed for TnI-Luc activity in both 23A2 and C2C12 myofibers. Reporter luciferase activity was normalized to the amount of *Renilla* luciferase activity and vehicle only was set to 100%. Data represents the mean and standard error of the mean (SEM) of three independent experiments. Different letters indicates a significant difference, $P<0.05$.

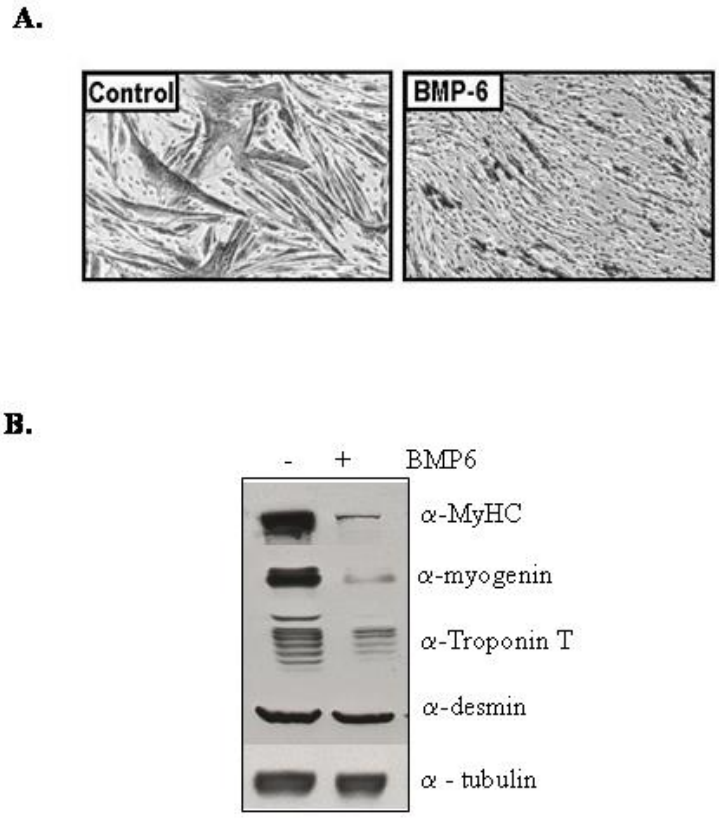
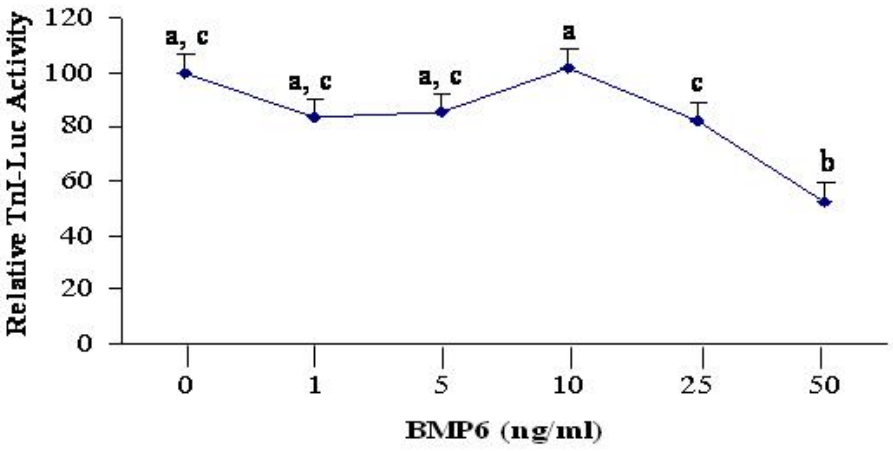


Figure 4-2. Inhibition of skeletal myogenesis by BMP6. C2C12 myoblasts were treated with 100 ng/ml BMP6 for 48 hours. Myoblasts were fixed and immunostained for MyHC expression. Representative microscopic images (200X) are shown (A). Parallel plates were lysed and analyzed for muscle specific proteins by Western blot (B). Tubulin expression was used as a loading control.

A.



B.

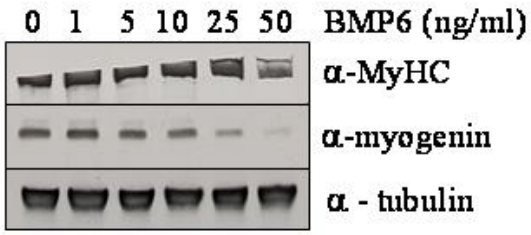


Figure 4-3. BMP6 dose response curve. C2C12 myoblasts ($1x10^5$) were transiently transfected with 2 μg of TnI-Luc reporter construct and 50 ng pRL-tk (*Renilla*). Cells were maintained in differentiation media supplemented with BMP6 for 48 hours. Cells were lysed and luciferase activities were measured (A). Reporter luciferase activity was normalized to the amount of *Renilla* luciferase activity and the control (vehicle only) was set to 100%. Data represents the mean and standard error of the mean (SEM) of three independent experiments. Different letters indicates significance at $p<0.05$. Total cell lysates were analyzed by Western blot for MyHC and myogenin protein expression (B). Tubulin expression was used as a loading control.

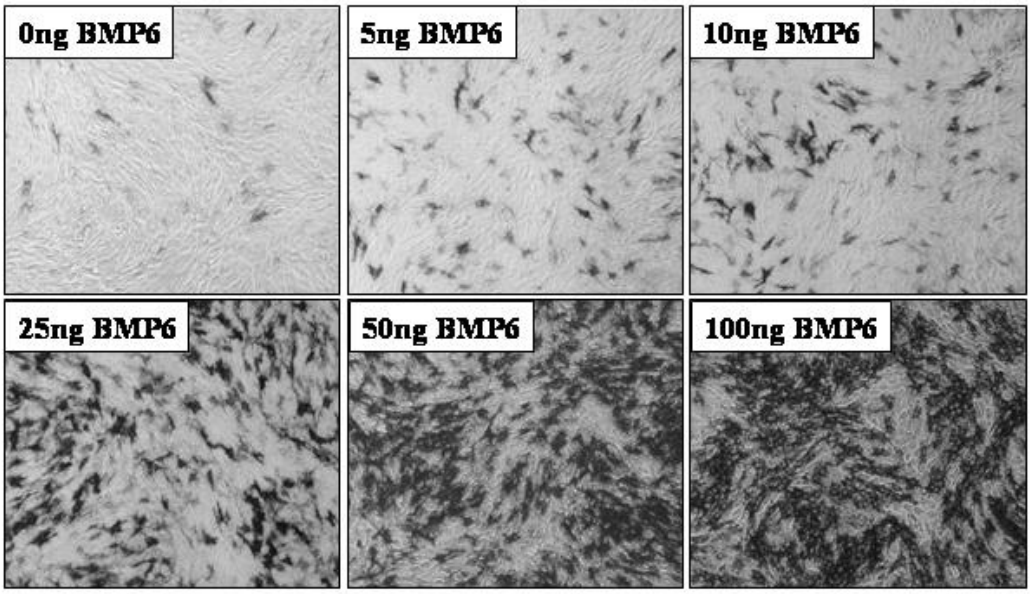


Figure 4-4. BMP6 induction of alkaline phosphatase activity. C2C12 myoblasts were treated with 0, 5, 10, 25, 50, and 100 ng/ml BMP6 for 48 hours. Cells were fixed and ALP activity detected colorimetrically. Representative photomicrographs at 200X are shown.

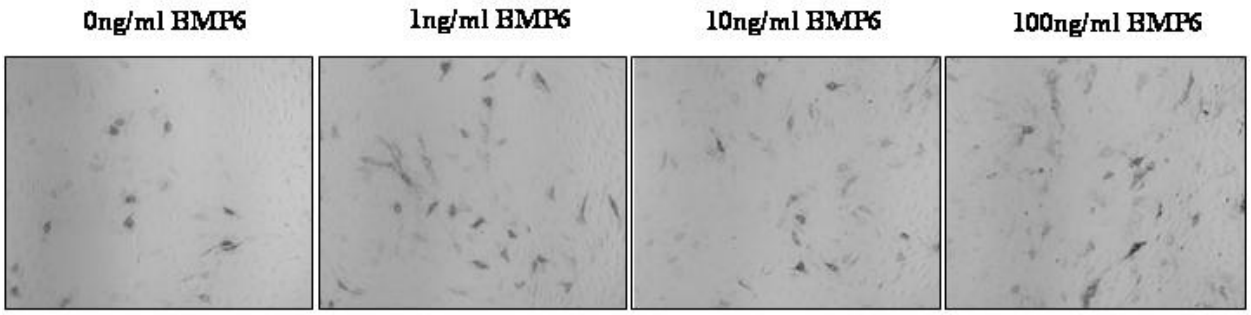


Figure 4-5. BMP6 does not induce alkaline phosphatase (ALP) activity in fibroblasts. C3H10T1/2 fibroblasts were treated with 0, 1, 10, and 100 ng/ml BMP6 for 48 hours. Cells were fixed, and stained for ALP activity. Representative photomicrographs at 200X are shown.

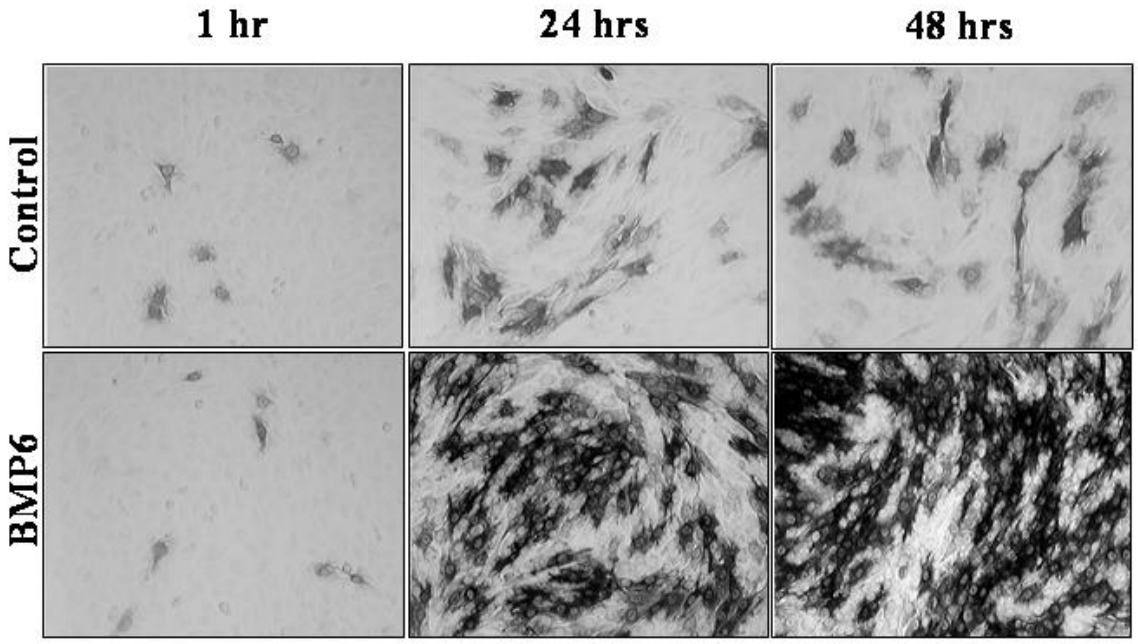


Figure 4-6. BMP6 induces rapid transdifferentiation in myoblasts. C2C12 myoblasts were treated for 1, 24 or 48 hours with vehicle alone (control) or 100 ng/ml BMP-6. Cultures were fixed with 4% paraformaldehyde and ALP activity measured colorimetrically. Representative photo-micrographs at 200X demonstrate intense ALP staining as early as 24 hours of treatment.

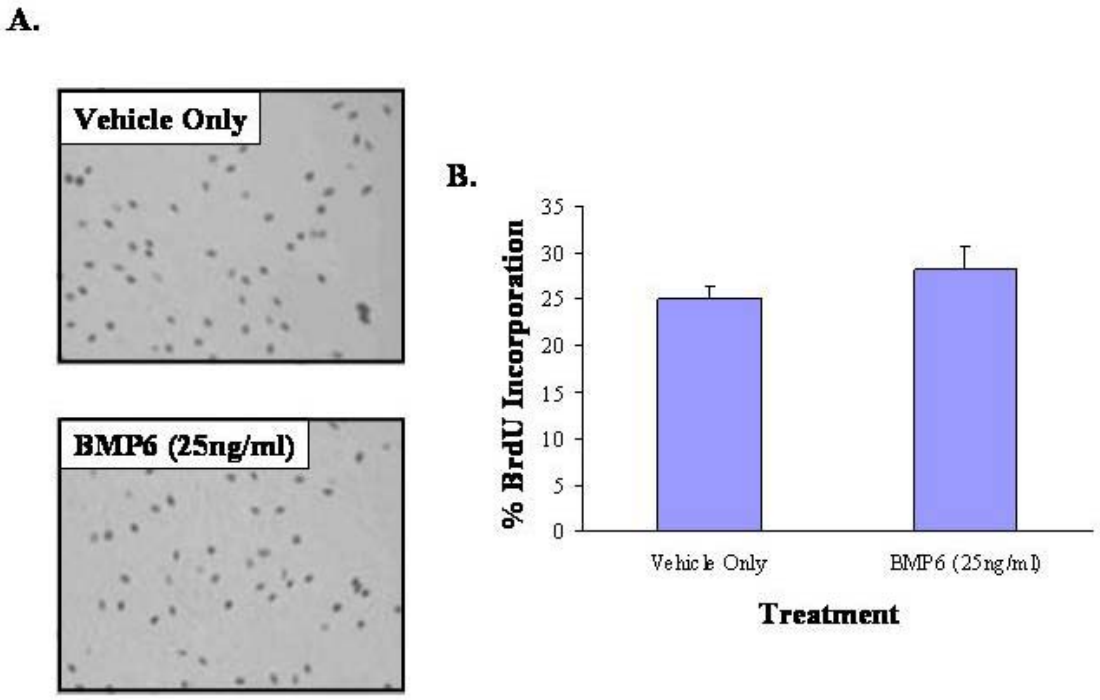


Figure 4-7. BMP6 treatment does not alter myoblast proliferation. C2C12 myoblasts were treated with 25 ng/ml BMP6 in differentiation medium for 48 hours, pulsed with 10 μM BrdU for 30 minutes, fixed with 70% ethanol at 4°C for 30 minutes and immunostained for BrdU expression (A). Representative photomicrographs at 100X shown. % BrdU incorporation was calculated by dividing the number of BrdU positive nuclei by the total number of nuclei (B). The averages of a minimum of six fields per treatment are shown.

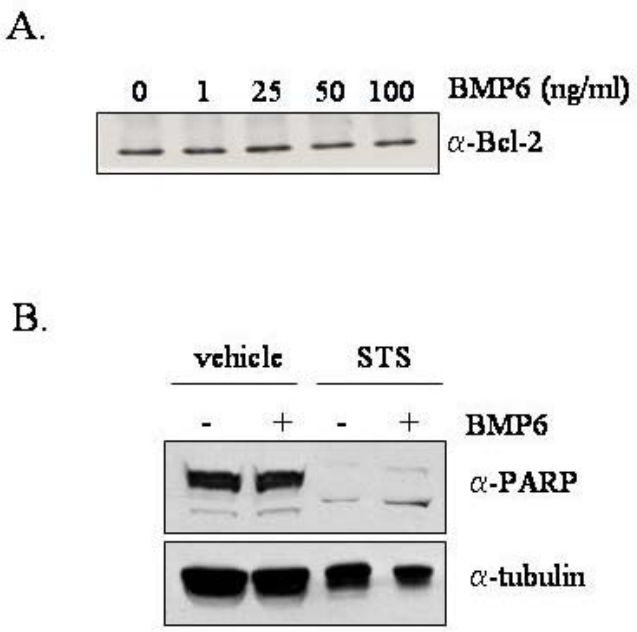


Figure 4-8. BMP6 is not anti-apoptotic. C2C12 myoblasts were placed in differentiation permissive media with increasing concentrations of BMP6 for 48 hours. Whole cell lysates were prepared and analyzed for expression of Bcl-2 by Western blot (A). C2C12 myoblasts were treated with 100ng/ml BMP6 in the absence or presence of 25 μM staurosporine (STS). Whole cell lysates were assessed for PARP cleavage and tubulin protein expression (B).

CHAPTER 5
BMP6 SIGNALING DURING SKELETAL MYOGENESIS

Objective

During skeletal myogenesis, BMP6 actions can be mediated by multiple signaling mechanisms (Figure 5-1). The specificity of the BMP6 and TGFβ_1 signaling responses are achieved by different types of Type I and Type II receptors and R-Smads (Wrana et al., 1992; Attisano et al., 1993; Ebner et al., 1993; Wieser et al., 1993; Wrana et al., 1994). Traditionally, BMPs signal through Smads 1/5/8, while TGFβ_1 signals through Smads 2/3. BMPs also initiate p38 signaling and within muscle, this regulation is specific to the p38α isoform. A third, more novel regulation, is through cross-talk with Notch signaling. The objective of these experiments was to validate the presence and activation of three putative intracellular signaling cascades induced by BMP6.

Analysis of BMP Signaling Systems in Myoblasts

The ability of BMP6 and TGFβ_1 to induce Smad phosphorylation and nuclear translocation was evaluated. In brief, C2C12 myoblasts were treated with 10 ng/ml TGFβ_1 and 25 ng/ml BMP6 for 48 hours. Total cell lysates were analyzed by Western blot for total and phospho-Smad1/5/8 expression. Myoblasts treated with vehicle alone demonstrated lows levels of phospho-Smad1/5/8, which were increased in response to both 10 ng/ml TGFβ_1 and BMP6 (Figure 5-2A). No differences in total Smad1/5/8 or tubulin were evident. Parallel plates of C2C12 myoblasts treated as described were fixed and immunostained for phospho-Smad1/5/8. In both instances, the signaling molecules are located in the nucleus (Figure 5-2B). Therefore, the archeotypical Smad signaling system is intact and functional in myoblasts. These results also support previous observations that demonstrated expression of Smad1/5/8 in C2C12 cells. Subsequent phosphorylation of Smad5 and weak phosphorylation of Smad1 was also observed

by BMP6. Smad8 was found to be constitutively phosphorylated in C2C12 cells (Ebisawa et al., 1999).

In addition to signaling through the Smad proteins, BMPs also may utilize components of the MAPK and Janus kinase (JAK)/signal transducers and activators of transcription (STAT) family of signaling proteins, culminating in activation of JNK or p38 (von Bubnoff and Cho, 2001). p38 signaling is a requirement for muscle formation (Zetser et al., 1999; Lee et al.,2002). The interplay of BMP6 signaling and p38 kinase activity during myogenesis was examined. C2C12 myoblasts were treated with increasing amounts of BMP6 and assessed for total and phospho-p38 protein expression by Western blot. Results demonstrate that while levels of total p38 don't change in response to BMP6, phospho-p38 is slightly induced in myoblasts treated with 25 and 50 ng/ml BMP6 (Figure 5-3).

C2C12 myoblasts were transiently transfected with TnI-Luc activity and treated with 100 ng/ml BMP6, 10 μM SB202190 or a combination of both for 48 hours. Results show 100 ng/ml BMP6 significantly inhibited TnI-Luc activity, as observed previously (Figure 5-4A, $P<0.05$). Treatment with 10 μM SB202190 inhibited TnI-Luc activity (Figure 5-4A, $P<0.05$). Importantly, the combination of BMP6 and SB202190 demonstrated an additive effect indicating independent pathways (Figure 5-4A, $P<0.0001$). Morphological observations showed myofibers in control cells, few myofibers in SB202190 treated, and no myofibers in either BMP6 treated or the combination of BMP6 and SB202190 (Figure 5-4B).

C2C12 myoblasts were treated with BMP6, SB202910, and BMP6 with SB202910 for 48 hours in differentiation media. Cells were lysed and assayed for MyHC, myogenin, Troponin T, and desmin protein expression by Western blot. Results demonstrate that muscle specific protein markers (MyHC, myogenin, and Troponin T) are reduced in the presence of BMP6, p38 inhibitor

(SB202910), and the combination (Figure 5-4). C2C12 myoblasts treated with both BMP6 and SB202910 demonstrated a more severe inhibition of myogenesis.

The importance of p38 signaling during BMP6-mediated transdifferentiation was examined. Parallel plates of C2C12 myoblasts treated with BMP6, SB202190, and the combination of BMP6 and SB202190. ALP activity was detected colorimetrically. Results show that inhibition of p38 function does not alter ALP induction in response to BMP6 treatment (Figure 5-6). This further demonstrates that BMP6 is signaling through an independent pathway to cause inhibition of differentiation and transdifferentiation.

Impact of Notch Inhibitor on BMP6-Mediated Inhibition of Differentiation

A third signaling mechanism used by BMP ligands involves Notch. Notch is a transmembrane receptor that is cleaved on the intracellular surface to release a proteolytic fragment that translocates to the nucleus and affects gene transcription (Nye et al., 1994; Ahmad et al., 1995). Notch inhibits myogenic differentiation similar to BMP6 (Kopan et al., 1994; Takahashi et al., 1994). BMP4 repression of muscle gene expression involves Notch activation (Dahlqvist et al., 2003). The involvement of BMP6 in Notch controlled signaling was examined in C2C12 myoblasts. In brief, myoblasts were induced to differentiate in the presence or absence of 25 ng/ml BMP6 and 10 μM L685,458, a Notch inhibitor, for 72 hours. Cells were fixed and immunostained for MyHC. Results show that MyHC was expressed in control cells and significantly inhibited in BMP6 treated (Figure 5-7A and 5-7B, $P<0.05$). Treatment of C2C12 myoblasts with L685,458 alone does not alter MyHC expression and interestingly, the combination of BMP6 and L685,458 partially restores MyHC expression (Figure 5-7A and 5-7B, $P<0.05$). This suggests that the BMP6-mediated inhibition of differentiation is partially controlled by the Notch signaling pathway.

In summary, differential BMP- and TGFβ-responsiveness and gene expression exists in myoblasts versus myofibers. This suggests that TGFβ superfamily members play different regulatory roles at various stages of myogenesis as myoblasts undergo terminal differentiation. Treatment of myoblasts with BMP6 results in a dramatic increase of ALP activity in a dose-dependent and time-dependent manner. Exogenous BMP6 treatment of skeletal myoblasts results in inhibition of myoblast differentiation as observed by significant inhibition of muscle reporter activity, muscle specific protein synthesis, and myoblast fusion. BMP6 treatment does not alter proliferation rates of myoblasts or promote a proliferative state to inhibit differentiation. Inhibition of p38 activity combined with BMP6 treatment caused inhibition of TnI-Luc activity that was greater than either treatment alone suggesting an additive effect between BMP6 and inhibition of p38 activity. Interestingly, the combination of BMP6 and Notch inhibition by L685,458 partially restores MyHC expression in fibers. This suggests that the myogenic inhibitory effect observed in the presence of BMP6 is partially mediated by functional Notch signaling.

Discussion

BMPs can be mediated by multiple signaling pathways and these experiments validated the presence and activation of these cascades in skeletal myoblasts. BMPs signal through serine/threonine kinase receptors (Massagué et al., 1994). In the presence of growth factors, ligands bind to a Type II receptor dimer located on the plasma membrane, which causes auto-phosphorylation of the Type II dimer and recruitment and transphosphorylation of a Type I receptor dimer (Wrana et al., 1992; Attisano et al., 1993; Ebner et al., 1993; Wieser et al., 1993; Wrana et al., 1994). This phosphorylation event recruits the receptor-regulated Smads (R-Smads), which then undergo phosphorylation (Aoki et al., 2001). The R-Smads form a complex

with the common-partner Smad (Co-Smad), Smad4. The R-Smad/Smad4 complex translocates into the nucleus and binds to DNA causing activation of target genes (Derynck et al., 1996; Liu et al., 1996; Meersseman et al. 1997; Nakao et al., 1997). Co-activators and co-repressors lend additional regulation to the system (Wotton et al., 1999). BMPs signal through Smads1/5/8 and the TGFβs typically signal through Smads2/3. BMP-activated Smads, most importantly Smad5, are necessary for inhibition of myogenic differentiation and osteoblastic induction in C2C12 cells (Lee et al., 2000). Assessment of functional Smad 1/5/8 activation was observed in the absence and presence of $TGF\beta_1$ or BMP6 treatment by immunostaining for phospho-Smad1/5/8 expression. Untreated C2C12 myofibers demonstrated low levels of phospho-Smad1/5/8 protein expression, which were increased in response to both 10 ng/ml $TGF\beta_1$ and 25 ng/ml BMP6 observed by Western analyses. Additionally, nuclear phospho-Smad1/5/8 protein expression was induced in response to 100 ng/ml BMP6 and 25 ng/ml $TGF\beta_1$ by immunoflourescence. Therefore, induction of phospho-Smad1/5/8 protein expression in response to BMP6 or $TGF\beta_1$ treatment demonstrates functional Smad1/5/8 activation in C2C12 myofibers and this induction of phospho-Smad1/5/8 is not due to an increase in total Smad1/5/8 protein expression.

Since both BMP6 and $TGF\beta_1$ induce functional Smad1/5/8 activation in myofibers, it may be argued that similar downstream effects could result from either ligand treatment. While BMP6 induces dramactic transdifferentiation of myoblasts, this same effect is not observed in response to $TG\beta_1$ treatment. Previous reports have demonstrated that $TGF\beta_1$ and BMP6 ligand bind to different Type II receptors and induce different target genes. For example, transfection of Smad1 and Smad5 into C2C12 myoblasts and NIH3T3 fibroblasts resulted in decreased myogenin promoter activity in both lines but ALP activity induction was only observed in C2C12 myoblasts and not in NIH3T3 fibroblasts. These results demonstrated that these

differentiation pathways are regulated independently at the transcriptional level (Yamamoto et al., 1997). Additionally, treatment of developing stage 24-25 chick limb buds with TGFβ_1 soaked beads resulted in dowregulation of *Indian hedgehog (ihh)*, *collagen type X (col X)* expression, which are markers of chondrocyte maturation, and further explain why BMP6 induces ALP activity but TGFβ_1 does not (Ferguson et al., 2004). Previous studies in the laboratory have also demonstrated that when TGFβ_1 biological activity was removed with an antibody that binds up endogenous TGFβ_1 ligand, restoration of the myogenic program is not restored, which suggests that TGFβ_1 is not targeting the myogenic differentiation program in the same manner as BMP6 (Wang et al., 2004). BMP6 also induces signaling ALP activity, which is not observed in response to TGFβ_1. TGFβ_1 does result in transdifferentiation of hepatic stellate cells to myofibroblasts and this process has actually been demonstrated to not be based on different regulation of Smad expression in these cells (Dooley et al., 2001). Therefore, even though BMP6 and TGFβ_1 induced the same Smads in myoblasts, the further downstream induction of gene targets is the critical mediator of the ultimate response and helps support why BMP6 causes transdifferentiation, but not TGFβ_1, in myoblasts.

BMPs can also initiate p38 signaling cascade and p38 is known to play a role in myogenic regulation. Most studies have shown that p38 signaling is a positive effector of skeletal myogenic differentiation and is required for myocyte formation and muscle-specific gene transcription. p38 MAPK can trigger cell cycle arrest during muscle differentiation (Lee et al., 2002). Other studies have demonstrated that interference of p38 activity with synthetic inhibitors abolish muscle cell fusion and expression of muscle-specific proteins (Zetser et al., 1999). Total p38 levels were unchanged in response to BMP6 treatment but phospho-p38 was slightly induced suggesting that low levels of p38 signaling may be active in myoblasts in response to

BMP6. Yet when myofibers were treated with a combination of 100 ng/ml BMP6 and a p38 inhibitor (SB202190), inhibition of p38 activity did not appear to alter ALP induction or inhibition of myofiber fusion in response to BMP6 treatment. Treatment of myofibers with BMP6 or SB202190 alone significantly inhibited TnI-Luc activity. The combination of 100 ng/ml BMP6 and 1 μM SB202190 further inhibited TnI-Luc activity as compared to either treatment alone. This demonstrates an additive effect of BMP6 and p38 inhibition on inhibition of TnI-luc reporter activity suggesting BMP6 and p38 are mediating inhibitory effects on skeletal myogenesis through independent pathways. Further support of independent signaling is the Western results demonstrating inhibition of both contractile and myogenic regulatory proteins by BMP6 and p38 independently and to different degrees. For example, BMP6 inhibits both contractile and regulatory proteins, whereas, p38 inhibition appears to target contractile proteins (MyHC). Furthermore, the complete ablation of muscle specific markers by BMP6 plus SB202901supports the additive effect demonstrated observed in the transient transfections on muscle specific reporter activity. The reason the phospho-p38 was slightly induced in response to BMP6 may be an antibody specificity issue. The antibody is supposed to be specific for only phospho-p38 but appears to be either picking up hypo-phosphorylated p38 or non-phosphorylated p38 at high levels of BMP6 treatment, which are not physiologically revelent dosages. Therefore, the activation of p38 signaling by BMP6 may not be relevant in an *in vivo* environment.

A third signaling pathway BMPs are regulated through is Notch signaling. Notch signaling plays a role in cellular homoestasis and cell fate determination and is important for proper cellular differentiation in many tissues (Hing et al., 1994). In mammals, there are four genes, Notch-1, Notch-2, Notch-3, and Notch-4 (Chitnis et al., 1995; Lindsell et al., 1995; Li et

al., 1998). The Notch receptors are transmembrane receptors and when bound by the extra-cellular ligands, Delta, Serrate, or Lag-2, this results in a cleavage of the intracellular domain of the receptors in the cytoplasm. This cleavage product is the active form of Notch, NICD (Notch Intracellular Domain), which translocates to the nucleus where it binds the family of transcriptional repressors, CSL (also known as RBP-Jκ, CBF-1, Suppressor of Hairless, and Lag-1), and converts them into transcriptional activators, which target genes involved in the inhibition of neurogenesis and myogenesis (Nye et al., 1994; Ahmad et al., 1995).

Functional Notch signaling is required for BMP4-mediated inhibition of differentiation of myogenic cells (Dahlqvist et al., 2003). Both Notch and BMP signaling can block differentiation of myogenic cells with ligand induction of Notch causing a dramatic block in myotube formation (Kopan et al., 1994; Takahashi et al., 1994; Kuroda et al., 1999). Therefore, to determine the involvement of Notch signaling in BMP6-mediated inhibition, Notch signaling was inhibited with L685,458 in the absence and presence of BMP6 in 23A2 myoblasts. Notch inhibition alone did not alter MyHC expression, although, BMP6 plus Notch inhibition did partially restore MyHC expression in fibers. This suggests that BMP6-mediated inhibition of differentiation is regulated or partially controlled by the Notch signaling pathway. Crosstalk could be further confirmed through utilization of a dominant negative version of the CSL, R218H. The cleavage product, NICD (Notch Intracellular Domain), is the active form of Notch, which translocates to the nucleus and binds the transcriptional repressors, CSL, converting them into transcriptional activators, which then target genes involved in the inhibition of myogenesis (Nye et al., 1994; Ahmad et al., 1995). CSL can also be thought of as the Notch signal mediator and R218H is thought to block activation of genes downstream of Notch by forming a complex with Notch ICD that cannot bind to the promoter (Chung et al., 1994; Wettstein et al., 1997). Transfection

of R218H alone and in combination with BMP6 in C2C12 and satellite cells would allow further confirmation of the Notch inhibitory effect of BMP6-mediated actions by determining MyHC, MyoD, myogenin, and Troponin T expression in response to the dominant negative CSL, R218H.

Analyses of Notch responsive genes and reporter activity would also demonstrate the importance of Notch signaling in BMP6-mediated myogenic inhibition. *Hes1* and *Hey1* are two immediate Notch responsive genes and could determine if BMP6 treatment of both satellite cells and C2C12 cells increased *Hes1* and *Hey1* expression by quantitative PCR. *Hey1* has been suggested to be important for inhibition of muscle development (Sun et al., 2001). Hey1-luc is a promoter construct that contains both CSL-binding sites and GC-rich domains (Kusanagi et al., 2000). Transfection of Hey1-luc into C2C12 and satellite cells and assessment of Hey1-luc activity in response to BMP6 and L685,458 alone and in combination would further demonstrate the role of Notch signaling in BMP6-mediate myogenic effects. The Notch and BMP signaling pathways are evolutionarily conserved and influence cellular differentiation in many tissues. Determination of how Notch and BMP6 signaling interact or crosstalk would contribute to further understanding of how cells respond to complex extracellular cues.

In summary, the functional role of BMP6 during myogenesis still remains unclear but these data provide further insight into the complex regulation of myogenesis mediated by BMP6. Functional Smad1/5/8 cascades are present in myoblasts, and BMP6-mediated myogenic inhibition is independent of p38 signaling but does partially require functional Notch sigaling. The requirement of additional factors required for this BMP6-mediated effect requires further evaluation. Activated Notch expression is highly localized expression to the site of injury suggesting that it might play a role in targeting or recruiting specific factors to the injury site to

promote regeneration. Interestingly, BMP6 is upregulated in response to wounding in keratinocytes (Wach et al., 2001). Therefore, due to the involvement of Notch in response to injury and BMPs utilization in many clinical studies for the generation of artificial tissues and various therapeutic interventions during bone injury repair, a more thorough understanding of their mechanism of action will assist in the development of therapeutics targeting muscle disease and repair.

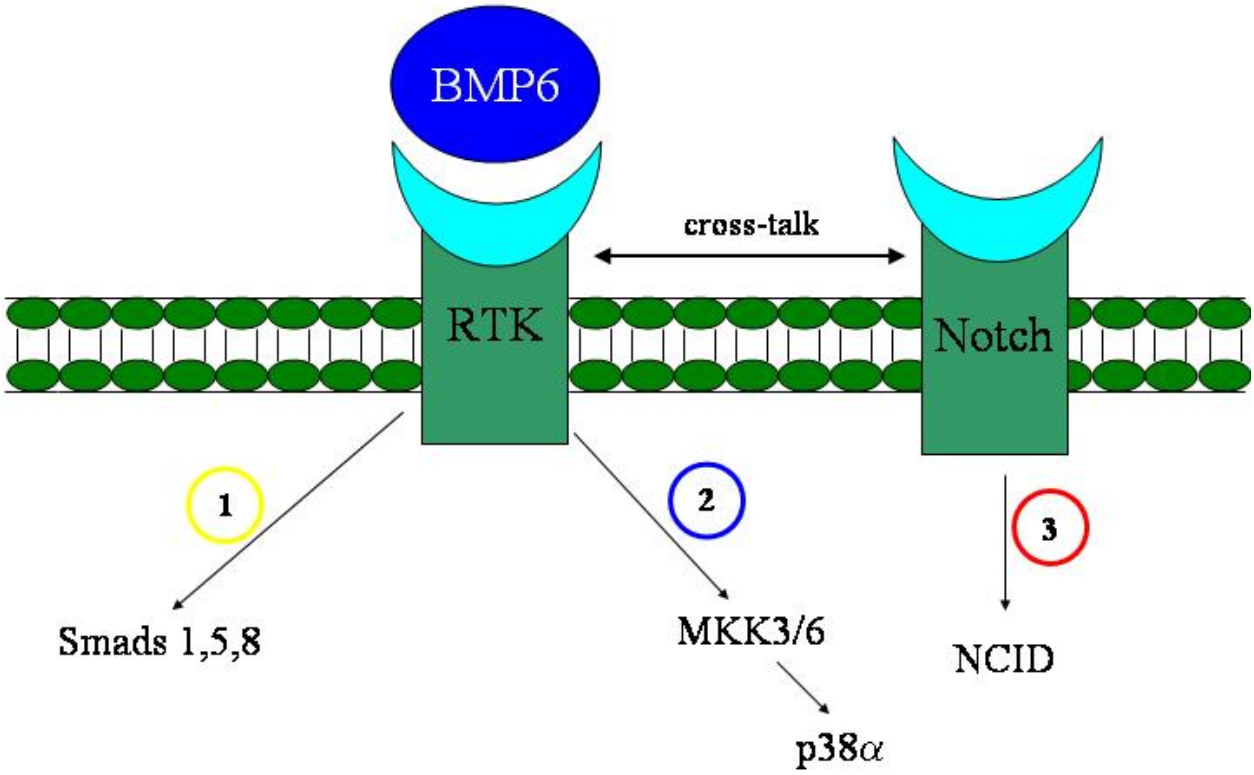


Figure 5-1. Potential BMP6 signaling pathways affecting skeletal myogenesis. Three intracellular signaling pathways highlight BMP6 signaling. Pathway#1 is the predominate Smad1/5/8 cascade; Pathway #2 is p38 signaling; Pathway #3 involves Notch signaling.

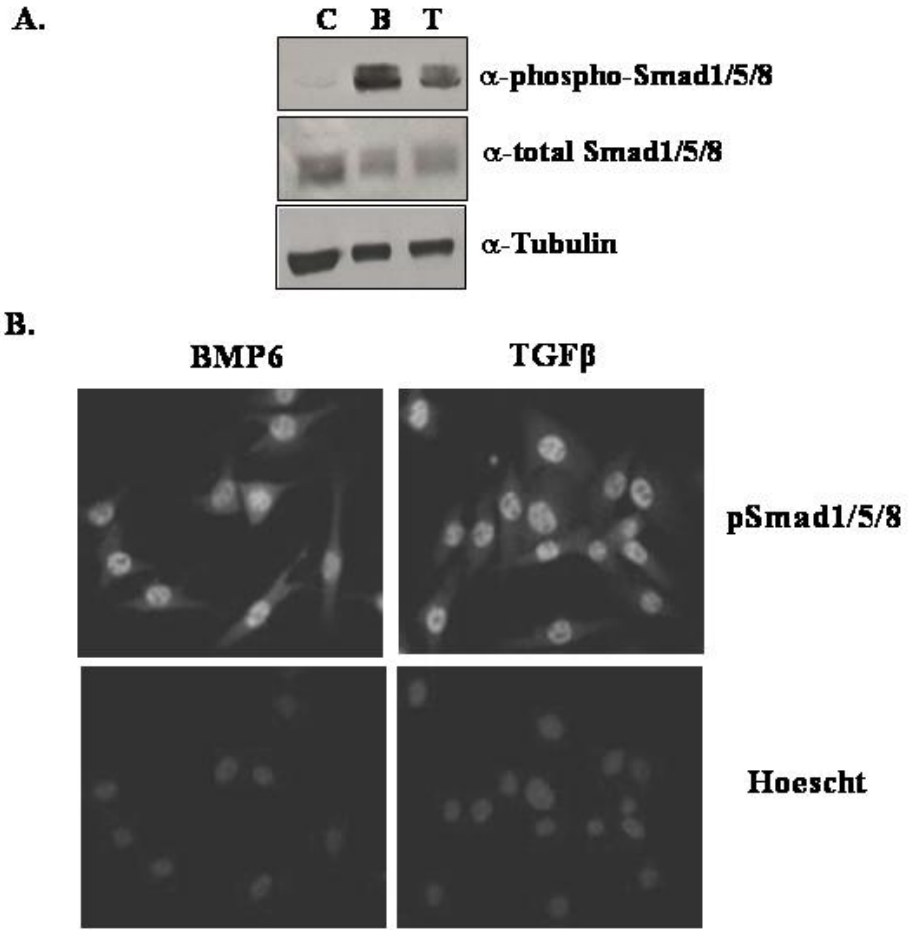


Figure 5-2. Verification of BMP and TGFβ signaling axis. C2C12 myoblasts were treated with 25 ng/ml BMP6 and 10 ng/ml $TGF\beta_1$. Parallel plates were immunostained for phospho-smad1/5/8 and Hoescht 33245 was used to visualize nuclei. Representative immunoflorescent images at 200X are shown (B). Total cell lysates were analyzed by Western blot for total Smad1/5/8 and phospho-Smad1/5/8 protein expression. Tubulin expression was used as a loading control (A).

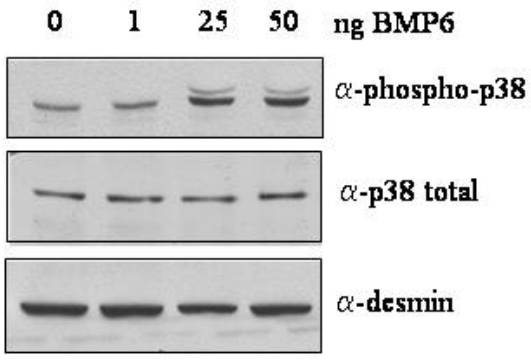


Figure 5-3. BMP6 treatment increases p38 phosphorylation. Cells were treated with increasing concentrations of BMP6 and total cell lysates were analyzed by Western blot for total and phospho-p38 protein expression. Desmin expression was used as a loading control.

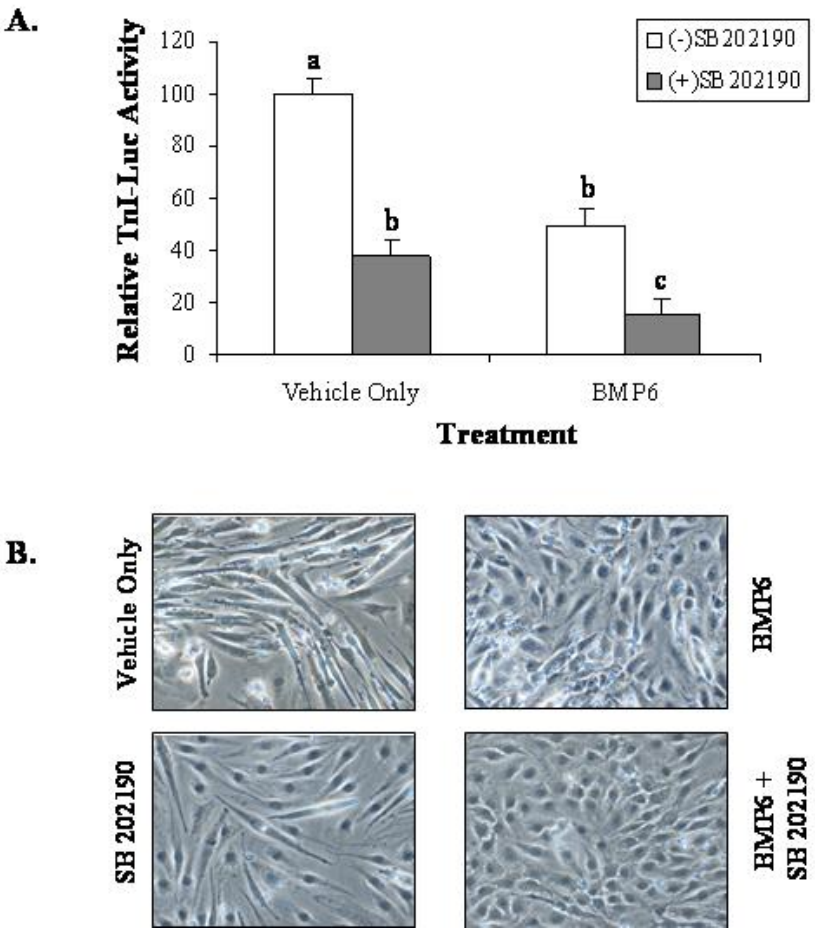


Figure 5-4. p38 inhibition and BMP6 treatment result in additive inhibition of muscle specific reporter activity. C2C12 myoblasts treated with 100 ng/ml BMP6 +/- 10 μM SB 202190 (p38 inhibitor) in differentiation media for 48 hours. Cells were lysed, and measured for TnI-Luc Activity (A). Reporter luciferase activity was normalized to the amount of *Renilla* luciferase activity and the control was set to 100% (vehicle only). Means and SEM are from three independent experiments. Different letters indicates significance at $P<0.05$. Representative phase contrast photomicrographs at 200X (B).

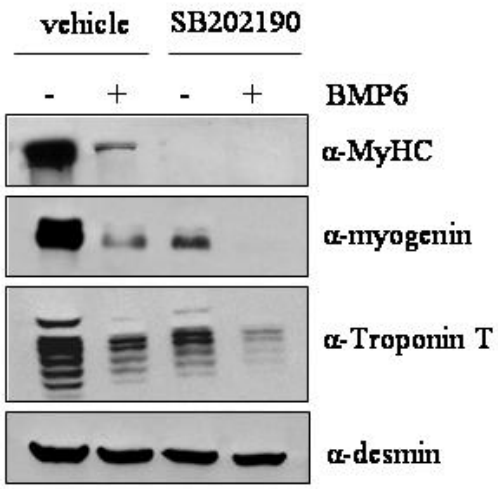


Figure 5-5. BMP6 and p38 signal through independent pathways to influence myogenic differentiation. C2C12 myoblasts were treated with 50 ng/ml BMP6 in the absence and presence of 10 μM SB202190 (p38 inhibitor) for 48 hours in differentiation permissive media. Cells were lysed and assessed for MyHC, myogenin, troponin T, and desmin protein expression by Western blot.

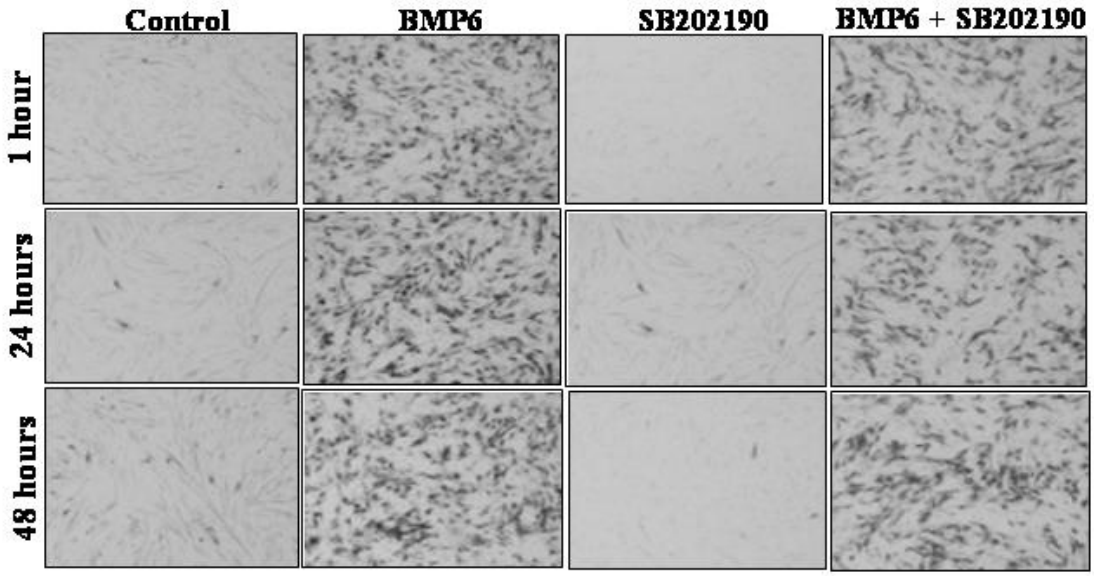


Figure 5-6. p38 signaling does not play a significant role in transdifferentiation. C2C12 myoblasts were treated with 100 ng/ml BMP6 +/- 10 μM SB 202190 (p38 inhibitor) for 48 hours. ALP activity was measured by reaction with NBT + BCIP colorimetrically. Representative photo-micrographs at 100X are shown.

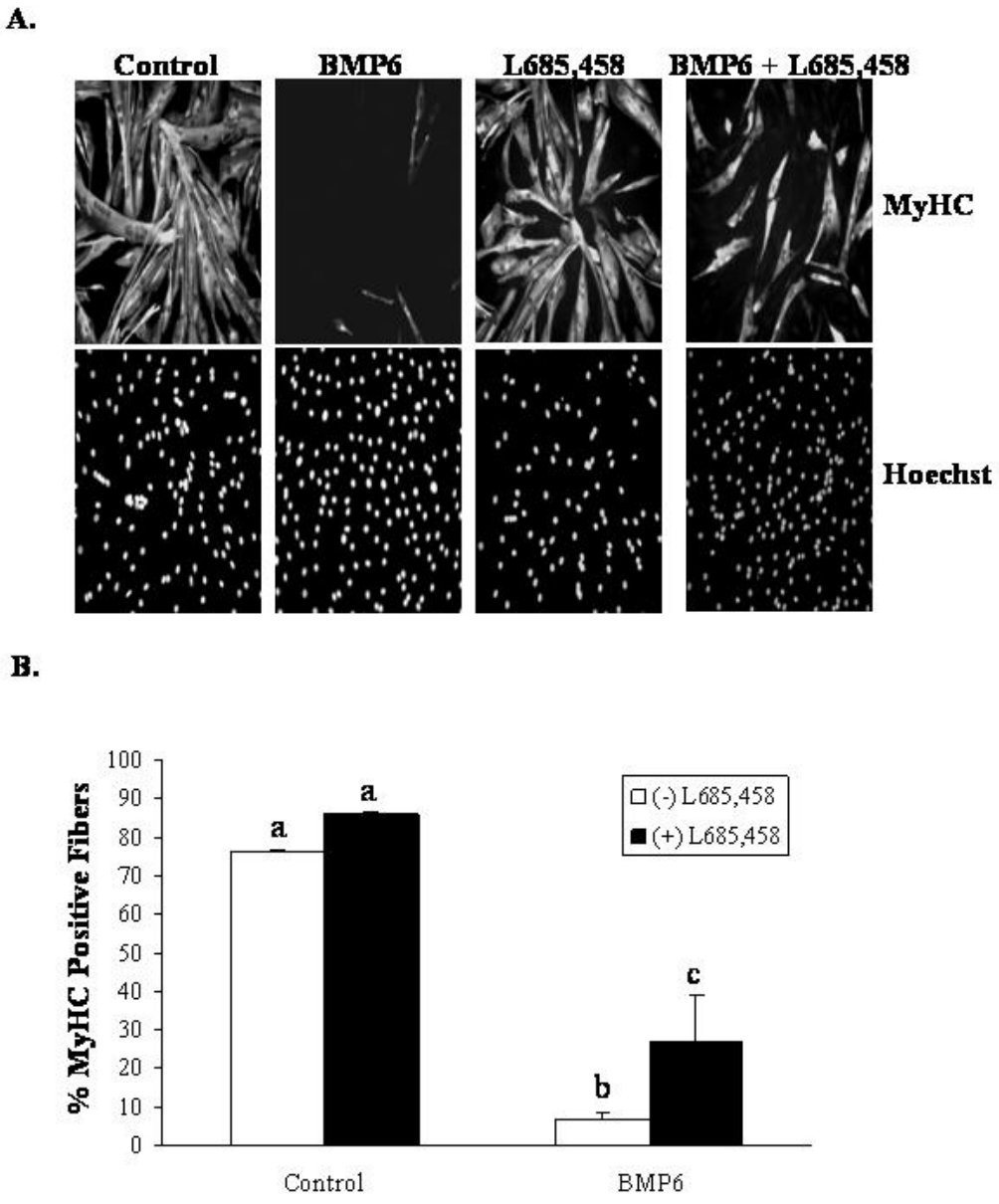


Figure 5-7. BMP6 inhibition of differentiation is mediated in part by Notch. C2C12 myoblasts were cultured with 25 ng/ml BMP6 +/- 1 μM L685,458 (Notch Inhibitor) for 72 hours. Cells were fixed and immunostained for myosin heavy chain (MyHC). Hoescht dye was used to visualize nuclei. Representative photomicrographs at 200X are shown (A). Quantification of the percentage of MyHC positive fibers in response to treatments (B). Different letters indicates a significant difference, $P<0.05$.

CHAPTER 6
IMPACT OF E2F5 ON SKELETAL MYOGENESIS

Objective

BMP6 expression is increased in Raf-arrested myocytes (Wang et al., 2004) and E2F5 is present in the nucleus of these Raf-arrested myoblasts (Reed et al., 2007). E2F5 is classified as a transcriptional repressor due in part to its negative regulation of cell cycle progression (DeGregori et al., 1997). In keratinocytes, BMP6 stimulates keratinocyte differentiation, which is associated with *E2F5*-upregulation and nuclear accumulation of E2F5 (D'Souza et al., 2001). The objective of these experiments was to examine the effect of E2F5 on skeletal myogenesis.

BMP6 Treatment does not cause E2F5 Nuclear Accumulation in Myoblasts

In keratinocytes, treatment with BMP6 significantly decreases DNA synthesis (D'Souza et al., 2001) and triggers differentiation programs (Tennenbaum et al., 1996). In these cultures, E2F5 protein levels are significantly increased (D'Souza et al., 2001). C2C12 myoblasts were treated with 25 ng/ml BMP6 for 48 hours, fixed and immunostained for E2F5 expression. Results demonstrate cytoplasmic retention of E2F5 in both control and BMP6 treated myoblasts (Figure 6-1). This indicates that BMP6 does not alter E2F5 translocation further supporting our contention that autocrine BMP6 does not participate in Raf-induced growth arrest.

Presence of E2F5 in Satellite Cell Position

E2F5 expression is found in the nucleus of quiescent cells *in vitro* and there also is a change in E2F expression with the onset of myoblast differentiation. Complexes of E2F5-p130 are present in G_0 phase but not throughout the remainder of the cell cycle (Olson et al., 1995). In rat cardiomyocytes, hypertrophic stimulus promotes *E2F1/3/4* and *DP1* expression while down-

regulating *E2F5*. These observations suggest that E2F5 plays a role in maintenance of growth arrest or quiescence (Hibi et al., 1996). Conversely, E2F5 expression is primarily in the cytoplasm of differentiated L6 myotubes, while pRb, p130, and p107 are present in the myonuclei suggesting cell type specificity (Epstein et al., 1995). To monitor the expression patterns of E2F5 in skeletal muscle, cryosections were collected form the tibialis anterior (TA) of adult male mice. The tissue was immunostained for E2F5 and dystrophin. As shown in Figure 6-2, E2F5 protein localizes to cells lying adjacent to the muscle fibers or putative satellite cells. Few E2F5 immunopositive nuclei were localized within the dystrophin boundary (Figure 6-2). Therefore, E2F5 is present in non-dividing, non-differentiating muscle cells *in vivo*.

E2F5 Does Not Inhibit Myofiber Differentiation

The presence of E2F5 in satellite cells suggests a role in cell cycle arrest and/or inhibition of differentiation. To determine if E2F5 was impacting subsequent myoblast fusion and differentiation, C3H10T1/2 fibroblasts were transiently co-transfected with TnI-Luc, pEM-MyoD, and CMV-E2F5. Cells were maintained in differentiation-permissive media for 48 hours prior to lysis and luciferase measurement. Muscle reporter gene activity was normalized to *Renilla* luciferase activity to account for transfection efficiency. C3H10T1/2 fibroblasts transfected with MyoD induced TnI-luc activity. Co-transfection of CMV-E2F5 did not significantly inhibit muscle specific reporter activity (Figure 6-3A). Therefore, E2F5 is not repressing myogenic gene transcription.

MyoD and E47 form heterodimers to enhance muscle gene transcription (Lassar et al., 1991). E47 plays a crucial role during skeletal myogenesis by serving as the preferred heterodimer binding partner of myogenin (Becker et al., 2001). C3H10T1/2 fibroblasts were transfected with an immunoglobulin E box reporter plasmid, μE5-luc, CMV-E47 and CMV-

E2F5. After 48 hours, cells were lysed and assayed for luciferase activity. Assessment of μE5-luc activity demonstrated that E47 function was not significantly affected by the addition of E2F5 (Figure 6-3B).

pRb does not interact with E2F5 to Exert Inhibitory Effects on Muscle Specific Activity

E2F gene activity is mediated through interaction with the pocket proteins, pRb, p107, and p130. E2F5 preferentially interacts with p130 and may require interaction with a pocket protein for a functional transcriptional effect. Previous work has demonstrated that only pRb translocates to the nucleus in response to Raf-induced quiescence (Reed, 2007). To determine if E2F5 requires pRb, myoblasts were transiently transfected with TnI-luc, CMV-E2F5, CMV-pRb, CMV-p107, and CMV-p130. Luciferase activity was measured after 48 hours. A reduction of TnI-Luc activity was observed in cells ectopically expressing p130 (Figure 6-4). Repression of reporter gene activity was not affected by E2F5. Therefore, the inability of E2F5 to inhibit myogenic differentiation is not a product of insufficient p130.

E2F5 is Transcriptionally Active

Since E2F5 did not cause an effect on myofiber differentiation, functional assessment of E2F5 activity was determined. C3H10T1/2 fibroblasts were transiently transfected with E2F-TA-luc, pRL-tk, and CMV-E2F5. Cells were lysed and assayed for luciferase activity. Both the complex thymidine kinase promoter reporter and the simple E2F *cis* element reporter were activated by E2F5 (Figure 6-5). These results imply that E2F5 is functional in myoblasts.

In summary, BMP6 treatment does not induce E2F5 nuclear accumulation. E2F5 does not inhibit myoblast differentiation. E2F5 does not appear to interact with pRb to mediate a transcriptional effect, although p130 does cause an inhibition of muscle reporter activity. Furthermore, the absence of a significant effect on myofiber differentiation was not due to an

inactive plasmid because the CMV-E2F5 was able to significantly induce E2F response reporters.

Discussion

Previous studies have demonstrated that E2F5 is necessary for pocket protein-mediated G_1 arrest in cycling cells (Gaubatz et al., 2000). E2F5 was believed to be involved in inhibition of the cell cycle in myoblasts, but in BMP6-treated myoblasts there was no induction of nuclear E2F5 accumulation in C2C12 myoblasts. This suggests that Raf signaling is not working through autocrine BMP6 nor is E2F5 activity directly mediated by BMP6.

Members of the E2F family are important in cell cycle progression and found to play roles in cellular processes like proliferation, differentiation, and apoptosis (Fujita et al., 2002). The E2F family regulates cell cycle progression and genes expressed at the G_1/S transition contain E2F binding sites within their promoters (Nevins, 1992; DeGregori et al., 1995). Two major groups, activators and repressors, comprise the E2F family. E2F1, E2F2, and E2F3 are involved in activating the cell cycle control and S-phase entry of quiescent cells. E2F4 and E2F5 are classified as repressor proteins, due to their inhibitory actions of cell cycle progression. E2F6, E2F7, and E2F8 are also classified as repressors, but the mechanism is independent of pocket protein interaction, demonstrating different regulatory mechanisms in comparison to the other E2F proteins (Campanero et al., 2000). Since *in vitro* studies have demonstrated E2F5 expression to be associated with quiescent cells and believed to play a role in maintenance of growth arrest or quiescence (Hibi et al., 1996), applicability of these observations need to be assessed in an *ex vivo* setting. Murine fibers isolated from retired breeders did demonstrate E2F5 expression in non-dividing muscle cells, which co-localized with Hoescht staining outside of the dystrophin border, demonstrating that active nuclear E2F5 expression is associated with quiescent muscle cells in the satellite cellular location. This further supports or mimics nuclear

E2F5 expression observed in Raf-arrested myoblasts and demonstrates that E2F5 is expressed in putative satellite cells *in vivo*.

E2F5 is expressed in the murine brain, heart, lung, liver, and kidney, and low levels in dermis and epidermis (D'Souza et al., 2001). The E2F5 knockout mouse is viable because it is believed that E2F4 and E2F5 have overlapping functions. The E2F5 knockout does develop hydrocephalus due to overproduction of cerebral spinal fluid resulting from a choroid plexus defect (Lindeman et al., 1998). The E2F4/5 double knockout is embryonic lethal (Gaubatz et al., 2000). Several labs have found E2F5 expressed in the nucleus, along with p130 in quiescent cells. E2F5 lacks a cyclin A binding domain, resulting in a shorter N-terminal as compared to other E2Fs and contains a C-terminal transactivation domain (Sardet et al., 1995).

Skeletal myoblasts undergo cell cycle withdrawal prior to subsequent differentiation and fusion. Since E2F5 is localized in putative satellite cells outside of the dystrophin border, it was believed that E2F5 may contribute to inhibition of differentiation. When C3H101T1/2 fibroblasts were transiently transfected with MyoD and assayed for muscle specific activity, TnI-luc activity was induced demonstrating proper myofiber differentiation. Co-transfection of MyoD and E2F5 did not alter TnI-luc induction suggesting that fibroblasts differentiation was not altered in the presence of E2F5 plasmid. Therefore, CH310T1/2 myofiber differentiation is not impacted by E2F5 expression assessed by TnI-luc activity and may be more crucial to maintaining growth arrest or quiescence of myoblasts.

Activation of muscle gene expression can be achieved through binding of Myogenic Regulatory Factors (MRF)-containing protein complexes to E-box elements within the regulatory regions of muscle specific genes (Johnson et al., 1996). *In vivo*, MRFs form heterodimers with members of the E protein family (E47, E2-2 or HEB), which are ubiquitously expressed bHLH

proteins and the molecular basis for inhibition of differentiation could involve direct modification of E-proteins (Shirakata et al., 1993). E47 is ubiquitously expressing in many cells including skeletal muscle cells and is an alternate splice product of the *E2A* gene (Murre et al., 1989; Sun and Baltimore, 1991). *E2A* knockout mice leads to subtle deficiencies in skeletal muscle function and null animals appear hunchback and are physically weak, suggesting that modest muscle defects exist (Bain et al., 1994; Yan et al., 1997). Luciferase analysis of fibroblasts demonstrated that E-box reporter activity is significantly stimulated by E47 yet the co-transfection of E47 with E2F5 is similar to E-box reporter activity observed in the presence of E47 alone demonstrating that E2F5 does not alter E-box reporter stimulation by E47. Therefore, the addition of E2F5 does not effect E47 stimulation of E-box reporter activity. These results demonstrate that E2F5 does not effect or inhibit myogenic differentiation. Previous reports demonstrate that Smad3 mediates myogenic differention in MyoD expressing C3H10T1/2 fibroblasts and C2C12 myoblasts by interacting with the HLH domain of MyoD/E protein heterodimerization. Smad3 interferes with the binding of MyoD complexes to E-box sites and results in inhibition of differentiation (Liu et al., 2001). Therefore, since BMP6 does not induced E2F5 nuclear accumulation and E2F5 does not impact E47 or E-box activity, BMP6-mediated inhibition of differentiation does not involve E2F5.

E2F5 is known to form a complex with pocket proteins, and interacts preferentially with p130 under physiological conditions to translocate to the nucleus for it to exert its transcriptional effect on target genes (Hijmans et al., 1995). C2C12 myoblasts were transiently transfected with E2F5 and pocket proteins, pRb, p107, and p130 along with TnI-luc reporter. Results demonstrate that the inability of E2F5 to inhibit differentiation is not due to insufficient pocket protein levels. Results also suggest that p130 alone is mediating an inhibitory effect on muscle

specific reporter activity in myoblasts. E2F5 stimulated transcription was found by other groups to be inhibited by cotransfection with p130, p107, and pRb in U2-OS osteosarcoma cells although this effect was DP-1 dependent (Hijmans et al., 1995). Previous studies in the laboratory have demonstrated that translocation of E2F5 to the nucleus in response to Raf is observed without subsequent p130 nuclear translocation. Although another report that overexpressed E2Fs in cardiomyocytes, which are also striated muscles, also demonstrated pocket proteins were not required (Ebelt et al., 2005). Alternatively, additional co-factors, such as HDAC1, may be necessary for nuclear translocation and repression of transcriptional events to occur. E2F5 has also been demonstrated to require dimerization with DP-1 to bind DNA suggesting that addition of DP-1 may have been needed for E2F5 to exert transcriptional activity (Hijmans et al,. 1995). Although, a lack of transcriptional effect by E2F5 on reporter activity is not due to an inactive plasmid because CMV-E2F5 was able to significantly induce a complete thymidine kinase promoter reporter, which is E2F responsive. E2F5 also significantly induced a simple E2F *cis* element reporter. This suggests that E2F5 is functional in myoblasts.

Since E2F5 is expressed in the satellite cell position but does not inhibit differentiation, creation of a stable myoblast line expressing a knockdown of E2F5 by siRNA or a dominant negative E2F5 (DN-E2F5) would further determine the role of E2F5 in skeletal myogenesis. If the cellular quiescence observed in Raf-arrested myoblasts were being mediated through E2F5, then the stable myoblasts with little to no E2F5 expression would not undergo this cell cycle arrest observed in the 23A2RafER^{DD} myoblasts. This would also demonstrate that E2F5 is responsible for maintenance of cellular quiescence of myoblasts. Conversely, assessment of a constitutively active E2F5 could be achieved by construction of a plasmid where the repressor domain was removed and replaced with a viral activator such as VP16. E2F5-VP16 could then

be utilized to create a stable myoblastic line that express constitutively active E2F5. In this line, one would expect that these lines would be in a state of continued cell cycle arrest and might demonstrate a phenotype similar to induced 23A2RafER^{DD} or Raf-arrested myoblasts and could serve as another model of quiescent skeletal myoblasts.

Assessment of signaling mechanisms and regulation events involved in skeletal myogenesis and further understanding of satellite cell activation will allow for better utilization of these muscle precursor cells in repairing and regenerating skeletal muscle. In addition, further insight into how myoblastic entrance and exit from the cell cycle is regulated and the specific gene expression patterns exhibited by quiescence myoblasts that are critical to the maintenance and regulation of the G_0 state. In conclusion, BMP6 does not induce E2F5 nuclear accumulation. E2F5 expresssion is localized in the putative satellite cell position *in vivo* in non-dividing muscle. E2F5 was not found to inhibit differentiation and this inability is not due to insufficient p130 or a non-functional plasmid. Therefore, due to the multipotency of satellite cells, applications in repair and regeneration of cartilage and bone might be further added by these data.

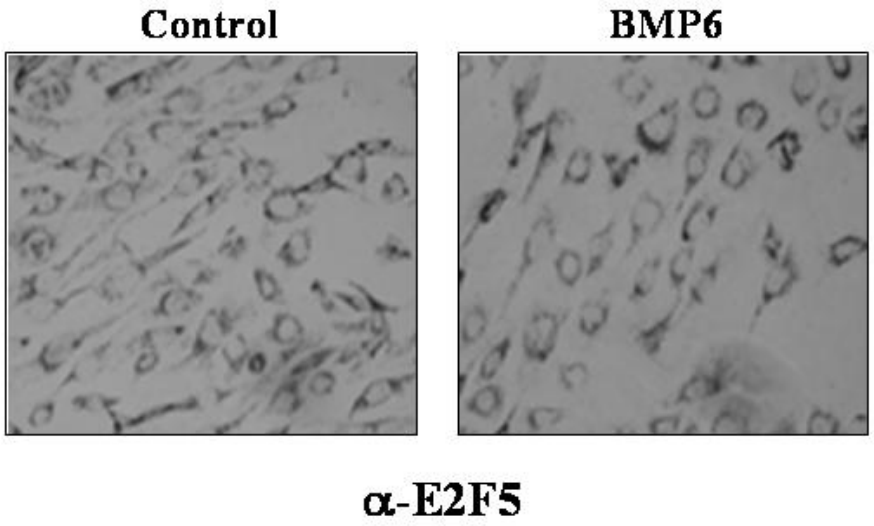


Figure 6-1. BMP6 treatment does not cause E2F5 nuclear accumulation in myoblasts. C2C12 myoblasts were treated with vehicle only (-) or 25 ng/ml BMP6 (+) for 48 hours and immunostained for E2F5. Representative photomicrographs at 200X are shown.

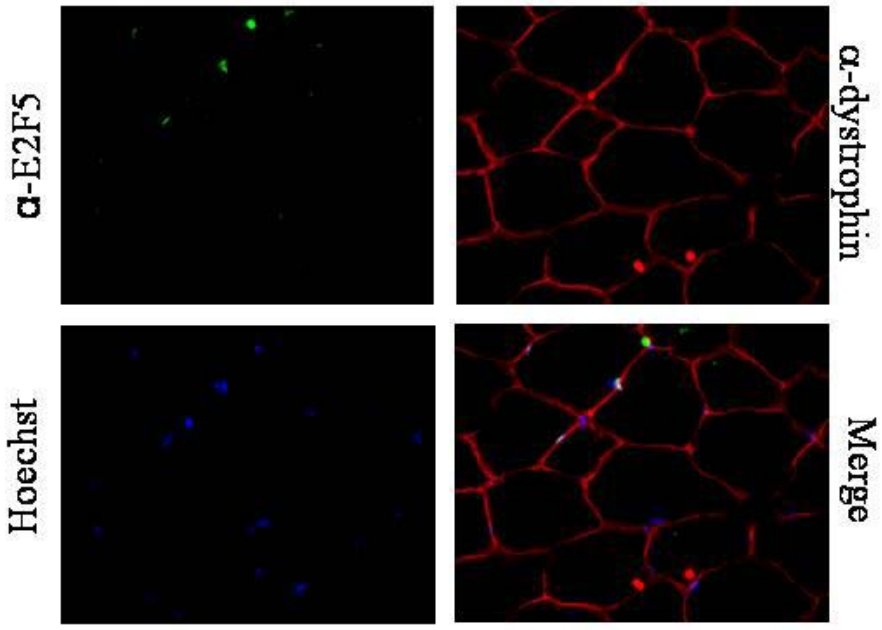


Figure 6-2. Presence of E2F5 in satellite cell position. Cryosections (10 μM) from murine TA muscle were immunostained for E2F5 and dystrophin. Hoechst 33245 was used as a nuclear counterstain. Representative photomicrographs at 200X are shown.

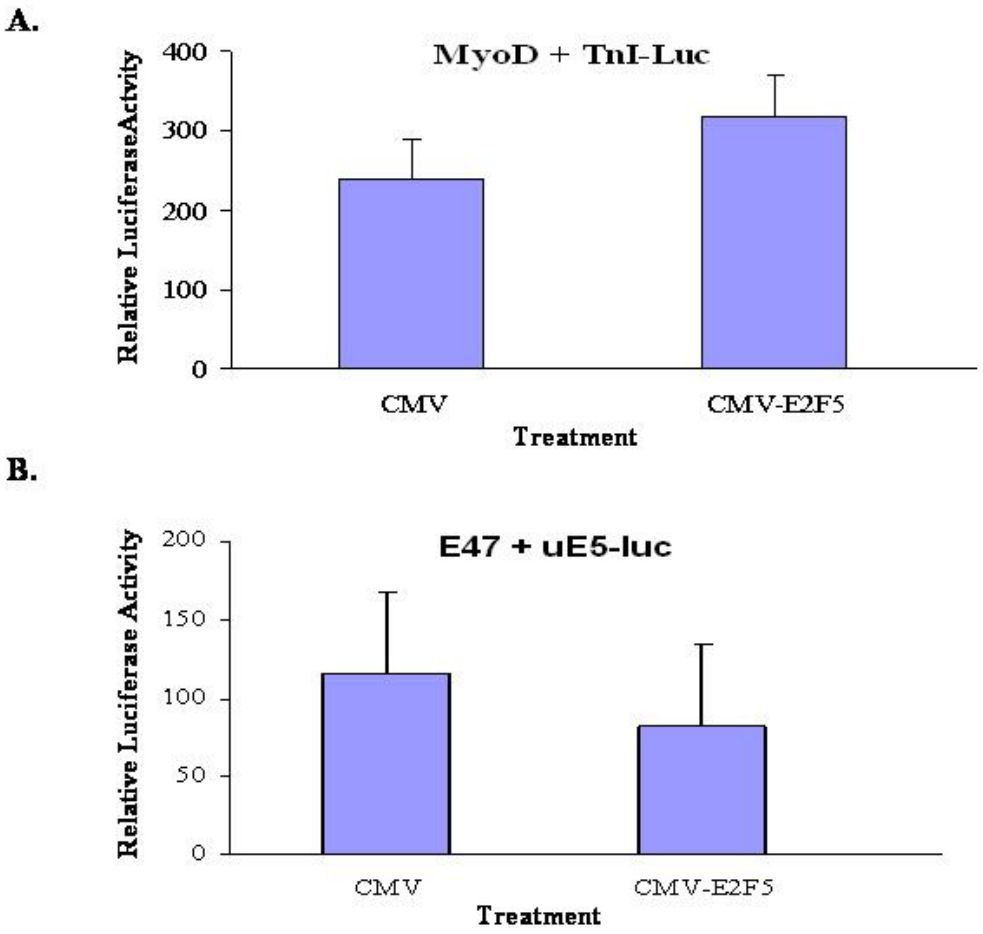


Figure 6-3. E2F5 does not inhibit myogenic differentiation. C3H10T1/2 fibroblasts were transiently transfected with pEM-MyoD, CMV-E2F5, and the muscle specific reporter (TnI-Luc) (A). Cells were transiently transfected with CMV-E47, CMV-E2F5, and the Immunoglobulin E box reporter plasmid μE5-Luc (B). Cells were lysed and luciferase activities measured. Reporter luciferase activity was normalized to the amount of *Renilla* luciferase activity. Data represents the mean and standard error of the mean (SEM) of three independent experiments.

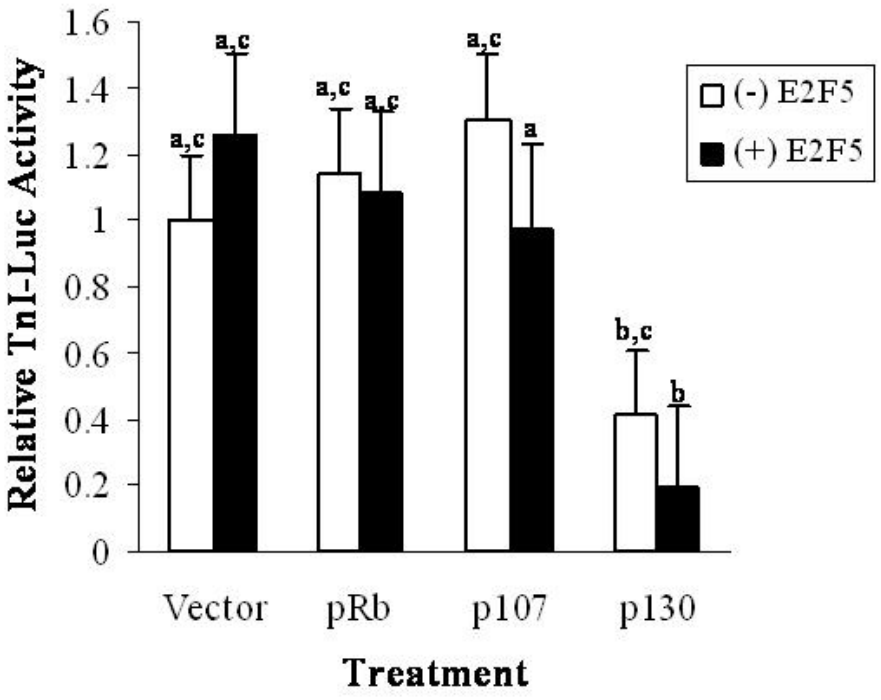


Figure 6-4. pRb does not interact with E2F5 to exert inhibitory effects on muscle specific activity. C2C12 myoblasts were transiently transfected with CMV-E2F5, pocket proteins (pRb, p107, and p130), and a TnI-Luc reporter. pRL-tk was included as a transfection efficiency monitor. Cells were lysed and luciferase measured. Reporter luciferase activity was normalized to the amount of Renilla luciferase activity. Data represents the mean and standard error of the mean (SEM) of three independent experiments. Different letters indicates a significant difference, $P<0.05$.

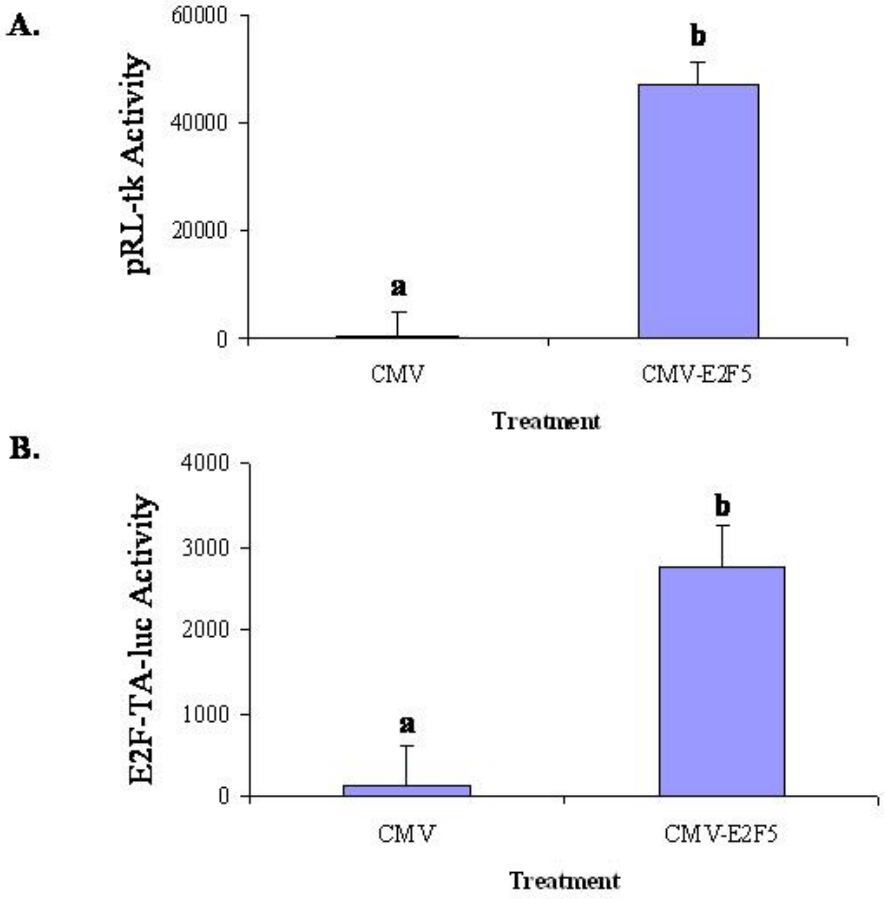


Figure 6-5. E2F5 is transcriptionally active. C3H10T1/2 fibroblasts were transiently transfected with pRL-tk, CMV-E2F5, and E2F-TA-luc reporter. Cells were lysed and luciferase activity measured. Data represents the mean and standard error of the mean (SEM) of three independent experiments. Different letters indicates a significant difference, $P<0.0001$.

CHAPTER 7
SUMMARY AND CONCLUSIONS

There appears to be differential gene expression and BMP- and TGFβ-responsiveness in embryonic myoblasts and adult satellite cells, as well as, different stages of skeletal myogenesis suggesting that different TGFβ superfamily members play different regulatory roles at various stages of muscle formation. *BMP6* was also found to be greater in myoblasts than myofibers but does not autocrinely regulate its own or other BMP ligands. The regulatory role of TGFβ superfamily member BMP6 on skeletal muscle differentiation was determined. Exogenous BMP6 results in inhibition of myoblastic differentiation as observed by significant inhibition of muscle reporter activity, muscle specific protein markers, and myoblast fusion. BMP6 also results in rapid transdifferentiation of myoblasts to an osteogenic cell lineage specific to mesodermally derived committed cells. Treatment of myoblasts with BMP6 does not alter the percentage of myoblasts in S-phase or promote a proliferative state to inhibit differentiation.

Induction of functional active phospho-Smad1/5/8 in response to BMP6 and TGFβ$_1$ was demonstrated by both immunofluorescence and Western blot analyses. Besides signaling through the Smad proteins, BMPs can also utilize components of the MAPK and JAK/STAT family, resulting in p38 activation (von Bubnoff and Cho, 2001). Inhibition of p38 activity and BMP6 treatment caused inhibition of TnI-Luc activity that was greater than either treatment alone suggesting an additive effect between BMP6 and p38 inhibition upon inhibition of myogenic differentiation. Results suggest that BMP6-mediated inhibition of differentiation is independent of p38 signaling.

BMPs can also interact with Notch signaling and previous studies have demonstrated that functional Notch signaling is required for BMP4-mediated inhibition of differentiation of muscle stem cells and C2C12 myogenic cells (Dahlqvist et al., 2003). Interestingly, the combination of

BMP6 and Notch inhibition by L685,458 partially restores MyHC expression in fibers. Therefore, BMP6-mediated inhibition of differentiation is regulated or partially controlled by the Notch signaling pathway similar to BMP4-mediated inhibition. Although, since MyHC expression is only partially restored there must also be additional factors mediating this inhibition of myogenic differentiation.

In summary, the functional role of BMP6 during myogenesis still remains unclear but these data provide further insight into the complex regulation of myogenesis mediated by BMP6. There does appear to be differences in expression of BMPs in myoblasts versus myofibers and BMP- and TGFβ-responsiveness. BMP6 inhibits the complete differentiation program in myoblasts. BMP6 does not appear to involve modulation of proliferation. BMP6 treatment results in a rapid transdifferentiation of myoblasts that is specific to a committed mesodermal derived myogenic cell. BMP6-mediated myogenic inhibition does partially require functional Notch sigaling. The requirement of additional factors required for this BMP6-mediated effect requires further evaluation. BMP6 is known to induce phospho-Smad1/5/8 and could potentially be forming a complex with NCID to bind to the promoter of muscle specific genes such as, MyHC to induced gene transcription required for myogenic differentiation (Figure 7-1).

Additionally, BMP6 expression is found to be increased in Raf-arrested myoblasts (Wang, 2004) and BMP6 is found to be expressed in mature muscle fibers (Dernyk, 1989). E2F5 is localized primarily in cytoplasm in proliferating myoblasts and is translocated primarily to the nucleus in response to elevated Raf expression. BMP6 stimulates keratinocyte differentiation associated with E2F5-upregulation (D'Souza et al., 2001). E2F5 is known to inhibit the cell cycle and is uniquely expressed in Raf-arrested myoblasts. The exit and entrance of skeletal myoblasts from the cell cycle and maintenance of cellular quiescence is an area of research not

well understood. In order to determine what role E2F5 plays in cellular quiescence of Raf-arrested myoblasts, BMP6 treatment was found to not induce E2F5 nuclear accumulation. E2F5 was found present in non-dividing cells *in vivo* in the satellite cellular position yet did not impact myogenic differentiation or E-box reporter activity. These results demonstrate that while E2F5 is localized in putative satellite cells outside the dystrophin border, E2F5 does not inhibit differentiation. Additionally, the inability of E2F5 to inhibit differentiation is not due to insufficient pocket proteins, although p130 appears to be inhibiting differentiation independently. Further understanding of the regulatory mechanisms involved in skeletal myogenesis and satellite cell activation will allow for determining better *in vitro* conditions of either injury or disease muscle models. Transcriptional modulation of processes such as differentiation and fusion could also be translated into *ex vivo* conditions for the prevention and treatment of skeletal muscle myopathies. Additionally, better utilization of muscle precursor cells and BMPs would prove applicable in the repair and regeneration of skeletal muscle, bone, and cartilage.

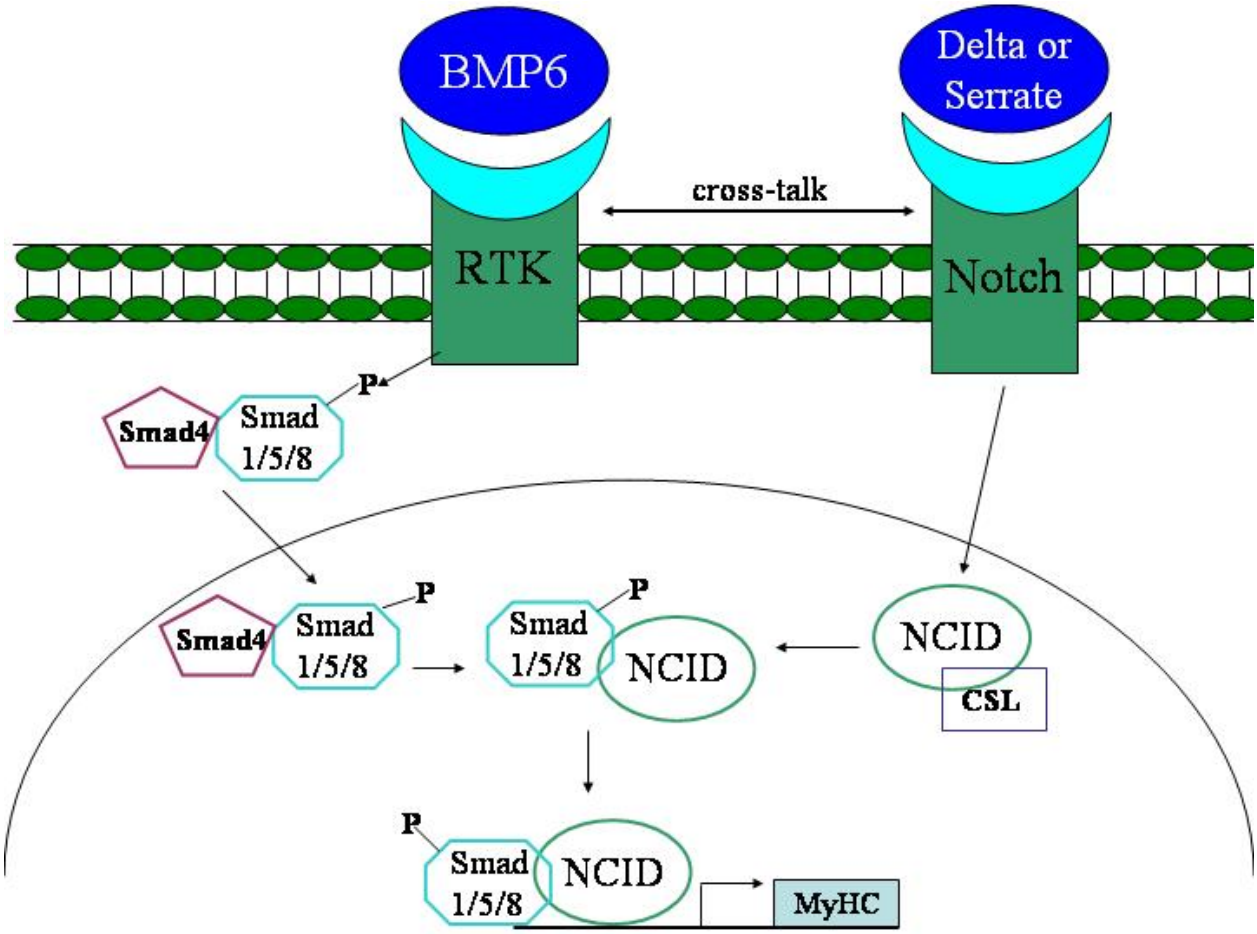


Figure 7-1. Proposed model of BMP6 on myogenic differentiation and interaction with Notch.

APPENDIX A
GENE ARRAY LAYOUT AND TABLE

Array Layout

Acvr1 1	Acvr1b 2	Acvr2a 3	Acvr2b 4	Acvrl1 5	Amh 6	Bambi 7	Bglap1 8
Bmp1 9	Bmp10 10	Bmp15 11	Bmp2 12	Bmp3 13	Bmp4 14	Bmp5 15	Bmp6 16
Bmp7 17	Bmp10 18	Bmp8b 19	Bmpr1a 20	Bmpr1b 21	Bmpr2 22	Cdc25a 23	Cdkn1a 24
Cdkn2b 17	Cer1 26	Chrd 27	Grem1 28	Col1a1 29	Col1a2 30	Col3a1 31	Dlx2 32
Lefty 33	Eng 34	Evi1 35	Fkbp1b 36	Fos 37	Fst 38	Gdf1 39	Gdf11 40
Gdf2 41	Gdf3 42	Gdf5 43	Gdf6 44	Gdf8 45	Gdf9 46	Id1 47	Id2 48
Id3 49	Id4 50	Cd79a 51	Igf1 52	Il6 53	Inha 54	Inhba 55	Inhbb 56
Inhbc 57	Inhbe 58	Itgb5 59	Itgb7 60	Ivl 61	Jun 62	Junb 63	Lap3 64
Lefty2 65	Igfbp3 66	Smad1 67	Smad2 68	Smad3 69	Smad5 70	Smad6 71	Smad7 72
Smad9 73	Nbl1 74	Nodal 75	Nog 76	Pdgfb 77	Plat 78	Plau 79	Runx1 80
Runx2 81	Serpine1 82	Sox4 83	Stat1 84	Tgfb1 85	Tgfb1i1 86	Tsc22d1 87	Tgfb2 88
Tgfb3 89	Tgfbi 90	Tgfbr1 91	Tgfbr2 92	Tgfbr3 93	Tgif 94	Timp1 95	Zfhx1a 96
PUC18 97	PUC18 98	PUC18 99	Blank 100	Blank 101	Blank 102	Gapdh 103	Gapdh 104
Ppia 105	Ppia 106	Ppia 107	Ppia 108	Rpl13a 109	Rpl13a 110	Actb 111	Actb 112

Gene Table

Position	Unigene	GeneBank	Symbol	Description	Gene Name
1	Mm.689	NM_007394	Acvr1	Activin A receptor, type 1	ALK2/ActR-I
2	Mm.308467	NM_007395	Acvr1b	Activin A receptor, type 1B	6820432J04/ActR-IB
3	Mm.314338	NM_007396	Acvr2a	Activin receptor IIA	ActRIIa/Acvr2
4	Mm.8940	NM_007397	Acvr2b	Activin receptor IIB	ActRIIB
5	Mm.279542	NM_009612	Acvrl1	Activin A receptor, type II-like 1	AI115505/AI427544

Position	Unigene	GeneBank	Symbol	Description	Gene Name
6	Mm.376094	NM_007445	Amh	Anti-Mullerian hormone	MIS
7	Mm.284863	NM_026505	Bambi	BMP and activin membrane-bound inhibitor, homolog (Xenopus laevis)	2610003H06Rik
8	Mm.87858	NM_007541	Bglap1	Bone gamma carboxyglutamate protein 1	Bglap/OC
9	Mm.27757	NM_009755	Bmp1	Bone morphogenetic protein 1	TLD
10	Mm.57171	NM_009756	Bmp10	Bone morphogenetic protein 10	BMP10
11	Mm.42160	NM_009757	Bmp15	Bone morphogenetic protein 15	AU015375/AU018861
12	Mm.103205	NM_007553	Bmp2	Bone morphogenetic protein 2	AI467020/Bmp2a
13	Mm.209571	NM_173404	Bmp3	Bone morphogenetic protein 3	9130206H07/9530029I04Rik
14	Mm.6813	NM_007554	Bmp4	Bone morphogenetic protein 4	Bmp2b/Bmp2b-1
15	Mm.118034	NM_007555	Bmp5	Bone morphogenetic protein 5	AU023399/se
16	Mm.374781	NM_007556	Bmp6	Bone morphogenetic protein 6	D13Wsu115e/Vgr-1
17	Mm.595	NM_007557	Bmp7	Bone morphogenetic protein 7	OP1
18	Mm.318417	NM_007558	Bmp8a	Bone morphogenetic protein 8a	Bmp7r1/OP-2
19	Mm.30413	NM_007559	Bmp8b	Bone morphogenetic protein 8b	Op3

Position	Unigene	GeneBank	Symbol	Description	Gene Name
20	Mm.237825	NM_009758	Bmpr1a	Bone morphogenetic protein receptor, type 1A	1110037I22Rik/ALK3
21	Mm.39089	NM_007560	Bmpr1b	Bone morphogenetic protein receptor, type 1B	AI385617/ALK-6
22	Mm.7106	NM_007561	Bmpr2	Bone morphogenic protein receptor, type II (serine/threonine kinase)	2610024H22Rik/AL117858
23	Mm.307103	NM_007658	Cdc25a	Cell division cycle 25 homolog A (S. cerevisiae)	D9Ertd393e
24	Mm.195663	NM_007669	Cdkn1a	Cyclin-dependent kinase inhibitor 1A (P21)	CAP20/CDKI
25	Mm.269426	NM_007670	Cdkn2b	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	AV083695/INK4b
26	Mm.6780	NM_009887	Cer1	Cerberus 1 homolog (Xenopus laevis)	Cerr1/cer-1
27	Mm.20457	NM_009893	Chrd	Chordin	Chd
28	Mm.166318	NM_011824	Grem1	Gremlin 1	Cktsf1b1/Drm
29	Mm.277735	NM_007742	Col1a1	Procollagen, type I, alpha 1	Col1a-1/Cola-1
30	Mm.277792	NM_007743	Col1a2	Procollagen, type I, alpha 2	AA960264/AI325291
31	Mm.249555	NM_009930	Col3a1	Procollagen, type III, alpha 1	AW550625/Col3a-1
32	Mm.3896	NM_010054	Dlx2	Distal-less homeobox 2	AW121999/Dlx-2
33	Mm.378911	NM_010094	Lefty1	Left right determination factor 1	AI450052/Leftb
34	Mm.225297	NM_007932	Eng	Endoglin	AI528660/CD105
35	Mm.56965	NM_007963	Evi1	Ecotropic viral integration site 1	D630039M04Rik/Evi-1

Position	Unigene	GeneBank	Symbol	Description	Gene Name
36	Mm.20453	NM_016863	Fkbp1b	FK506 binding protein 1b	AW494148
37	Mm.246513	NM_010234	Fos	FBJ osteosarcoma oncogene	D12Rfj1/c-fos
38	Mm.4913	NM_008046	Fst	Follistatin	FST
39	Mm.258280	NM_008107	Gdf1	Growth differentiation factor 1	AI385651/Gdf-1
40	Mm.299218	XM_125935	Gdf11	Growth differentiation factor 11	Bmp11
41	Mm.343728	NM_019506	Gdf2	Growth differentiation factor 2	Bmp9
42	Mm.299742	NM_008108	Gdf3	Growth differentiation factor 3	C78318/Gdf-3
43	Mm.4744	NM_008109	Gdf5	Growth differentiation factor 5	CDMP-1/bp
44	Mm.302555	NM_013526	Gdf6	Growth differentiation factor 6	BMP13/GDF16
45	Mm.3514	NM_010834	Gdf8	Growth differentiation factor 8	Cmpt/Mstn
46	Mm.9714	NM_008110	Gdf9	Growth differentiation factor 9	Gdf-9
47	Mm.444	NM_010495	Id1	Inhibitor of DNA binding 1	AI323524/D2Wsu140e
48	Mm.34871	NM_010496	Id2	Inhibitor of DNA binding 2	AI255428/C78922
49	Mm.110	NM_008321	Id3	Inhibitor of DNA binding 3	HLH462/Idb3
50	Mm.283273	NM_031166	Id4	Inhibitor of DNA binding 4	Idb4
51	Mm.1355	NM_007655	Cd79a	CD79A antigen (immunoglobulin-associated alpha)	Ig-alpha/Iga
52	Mm.268521	NM_010512	Igf1	Insulin-like growth factor 1	C730016P09Rik/Igf-1
53	Mm.1019	NM_031168	Il6	Interleukin 6	Il-6
54	Mm.1100	NM_010564	Inha	Inhibin alpha	AW555078

Position	Unigene	GeneBank	Symbol	Description	Gene Name
55	Mm.8042	NM_008380	Inhba	Inhibin beta-A	INHBA
56	Mm.3092	NM_008381	Inhbb	Inhibin beta-B	INHBB
57	Mm.2594	NM_010565	Inhbc	Inhibin beta-C	INHBC
58	Mm.3510	NM_008382	Inhbe	Inhibin beta E	INHBE
59	Mm.6424	NM_010580	Itgb5	Integrin beta 5	AA475909/AI874634
60	Mm.58	NM_013566	Itgb7	Integrin beta 7	Ly69
61	Mm.207365	NM_008412	Ivl	Involucrin	1110019C06RIK
62	Mm.275071	NM_010591	Jun	Jun oncogene	AP-1/Junc
63	Mm.1167	NM_008416	Junb	Jun-B oncogene	JUNB
64	Mm.286830	NM_024434	Lap3	Leucine aminopeptidase 3	2410015L10Rik/AA410100
65	Mm.87078	NM_177099	Lefty2	Left-right determination factor 2	6030463A22Rik/AV214969
66	Mm.29254	NM_008343	Igfbp3	Insulin-like growth factor binding protein 3	AI649005/IGFBP-3
67	Mm.223717	NM_008539	Smad1	MAD homolog 1 (Drosophila)	AI528653/Madh1
68	Mm.152699	NM_010754	Smad2	MAD homolog 2 (Drosophila)	Madh2/Madr2
69	Mm.7320	NM_016769	Smad3	MAD homolog 3 (Drosophila)	AU022421/Madh3
70	Mm.272920	NM_008541	Smad5	MAD homolog 5 (Drosophila)	1110051M15Rik/AI451355
71	Mm.325757	NM_008542	Smad6	MAD homolog 6 (Drosophila)	Madh6
72	Mm.34407	NM_008543	Smad7	MAD homolog 7 (Drosophila)	Madh7
73	Mm.244353	NM_019483	Smad9	MAD homolog 9 (Drosophila)	MADH6/Madh9
74	Mm.9404	NM_008675	Nbl1	Neuroblastoma, suppression of tumorigenicity 1	D4H1S1733E/DAN
75	Mm.57195	NM_013611	Nodal	Nodal	Tg.413d
76	Mm.135266	NM_008711	Nog	Noggin	NOG
77	Mm.144089	NM_011057	Pdgfb	Platelet derived growth factor, B polypeptide	PDGF-B/Sis
78	Mm.154660	NM_008872	Plat	Plasminogen activator, tissue	AU020998/AW212668
79	Mm.4183	NM_008873	Plau	Plasminogen activator, urokinase	u-PA/uPA

Position	Unigene	GeneBank	Symbol	Description	Gene Name
80	Mm.4081	NM_009821	Runx1	Runt related transcription factor 1	AI462102/AML1
81	Mm.263975	NM_009820	Runx2	Runt related transcription factor 2	AML3/Cbf
82	Mm.250422	NM_008871	Serpine1	Serine (or cysteine) peptidase inhibitor, clade E, member 1	PAI-1/PAI1
83	Mm.240627	NM_009238	Sox4	SRY-box containing gene 4	AA682046/Sox-4
84	Mm.277406	NM_009283	Stat1	Signal transducer and activator of transcription 1	2010005J02Rik/AA408197
85	Mm.248380	NM_011577	Tgfb1	Transforming growth factor, beta 1	TGF-beta1/Tgfb
86	Mm.3248	NM_009365	Tgfb1i1	Transforming growth factor beta 1 induced transcript 1	ARA55/Hic5
87	Mm.153272	NM_009366	Tsc22d1	TSC22 domain family, member 1	AA589566/AW105905
88	Mm.18213	NM_009367	Tgfb2	Transforming growth factor, beta 2	BB105277/Tgf-beta2
89	Mm.3992	NM_009368	Tgfb3	Transforming growth factor, beta 3	Tgfb-3
90	Mm.14455	NM_009369	Tgfbi	Transforming growth factor, beta induced	68kDa/AI181842
91	Mm.197552	NM_009370	Tgfbr1	Transforming growth factor, beta receptor I	ALK5/AU017191
92	Mm.172346	NM_009371	Tgfbr2	Transforming growth factor, beta receptor II	1110020H15Rik/AU042018
93	Mm.200775	NM_011578	Tgfbr3	Transforming growth factor, beta receptor III	1110036H20Rik/AU015626
94	Mm.101034	NM_009372	Tgif	TG interacting factor	AA959811/AI462167

Position	Unigene	GeneBank	Symbol	Description	Gene Name
95	Mm.8245	NM_011593	Timp1	Tissue inhibitor of metalloproteinase 1	Clgi/TIMP-1
96	Mm.3929	NM_011546	Zfhx1a	Zinc finger homeobox 1a	3110032K11Rik/AREB6
97	N/A	L08752	PUC18	PUC18 Plasmid DNA	pUC18
98	N/A	L08752	PUC18	PUC18 Plasmid DNA	pUC18
99	N/A	L08752	PUC18	PUC18 Plasmid DNA	pUC18
100					
101					
102					
103	Mm.343110	NM_008084	Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	Gapd
104	Mm.343110	NM_008084	Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	Gapd
105	Mm.5246	NM_008907	Ppia	Peptidylprolyl isomerase A	2700098C05/Cphn
106	Mm.5246	NM_008907	Ppia	Peptidylprolyl isomerase A	2700098C05/Cphn
107	Mm.5246	NM_008907	Ppia	Peptidylprolyl isomerase A	2700098C05/Cphn
108	Mm.5246	NM_008907	Ppia	Peptidylprolyl isomerase A	2700098C05/Cphn
109	Mm.180458	NM_009438	Rpl13a	Ribosomal protein L13a	1810026N22Rik/Tstap198-7
110	Mm.180458	NM_009438	Rpl13a	Ribosomal protein L13a	1810026N22Rik/Tstap198-7
111	Mm.297	NM_007393	Actb	Actin, beta, cytoplasmic	Actx/E430023M04Rik
112	Mm.297	NM_007393	Actb	Actin, beta, cytoplasmic	Actx/E430023M04Rik

APPENDIX B
SUMMARY OF ABBREVIATIONS

BMPs: Bone Morphogenetic Proteins

SR: sarcoplasmic reticulum

T system: Transverse Tubule system

IF: Intermediate Filaments

RyR: ryanodine receptors

DHP: dihydropyridine receptors

MyHC: Myosin Heavy Chain

MPCs: Muscle Precursor Cells

MRFs: Myogenic Regulatory Factors

NMJ: neuromuscular junction

MNF: Myocyte Nuclear Factor

HGF: Hepatocyte Growth Factor

TGFβ: Transforming Growth Factor Beta

FGF: Fibroblast Growth Factor

IGF: Insulin Growth Factor

PI3K: Phosphatidylinositol-3-kinase

TA: Tibialis anterior

MSTN: Myostatin

ATP: adenosine tri-phosphate

ADP: adenosine di-phosphate

VO_2 max: maximal oxygen uptake

dpc: days post coitum

Tn: Troponin

Id: inhibitor of differentiation/DNA binding

GDF: growth and differentiation factor

MAPK: mitogen-activated protein kinase

JNK: Jun N-terminal kinase

bHLH: basic helix-loop-helix

STAT: signal transducers and activators of transcription

MEK: mitogen-activated protein kinase/extracellular signal regulated kinase kinase

MEKK: MEK kinase

ERK: extracellular regulated signal kinase

G_0: quiescence or cell cycle arrest

MEF: myocyte enhancer-binding factor

cdk: cyclin-dependent kinase

S-phase: synthesis phase

MSCs: Mesenchymal stem cells

PTH: parathyroid hormone

PCNA: proliferating cell nuclear antigen

NICD: Notch Intracellular Domain

CSL: RBP-Jκ or CBF-1, Suppressor of Hairless, and Lag-1

Mdx: dystrophin null transgenic mice

CME: crushed muscle extract

Rb: retinoblastoma

Shh: Sonic hedgehog

Ihh: Indian hedgehod

ColX: collagen type X

ROM: range of motion

PCSA: physiological cross-sectional area

PCR: polymerase chain reaction

RT: reverse transcription

IHC: immunohistochemical

FACS: Fluorescence Activated Cell Sorting

DAPI: 4,6-diamidino-2-phenylinodale

4HT: 4-hydroxytamoxifen

FBS: fetal bovine serum

PBS: phosphate buffered saline

TnI-luc: Troponin I luciferase

GAPDH: glyceraldehydes-3-phosphate dehydrogenase

tk: thymidine kinase

ER: estrogen receptor

DBD: DNA binding domain

EtOH: ethanol

HS: horse serum

HCl: Hydrochloric acid

DAB: 3,3'-Diaminobenzidine tetrahydrochloride

NiCl: Nickel Chloride

H_2O_2: hydrogen peroxide

BSA: bovine serum albumin

BCIP: 5-bromo-4-chloro-3-indolyl-phosphate

NBT: Nitro Blue Tetrazolium

PARP: anti-Poly (ADP-ribose) polymerase

AP: Alkaline phosphatase-conjugated streptavidin

ALP: alkaline phosphate

SDS: sodium dodecyl sulfate

SSC: saline sodium citrate

O/N: overnight

Bmpr: Bone Morphogenetic Protein Receptor

Col1a1: Procollagen, type I, alpha 1

Itgb5: Integrin beta 5

Igfbp3: Insulin-like growth factor binding protein 3

Runx: Runt related transcription factor

myogenin-CAT: myogenin/chloramphenical acetyltransferase

ECM: extracellular matrix

PCP: procollagen C proteinase

ALK: activin receptor-like kinase

APAF-1: apoptotic protease activign factor 1

TNF: Tumor Necrosis Factor

FADD: Fas-associated death domain

STS: staurosporine

JAK: Janus kinase

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BIOGRAPHICAL SKETCH

Jennelle Robin McQuown was born in Oswego, NY to Dan and Linda McQuown. She is the middle child of three, having one older brother, Dan and a younger sister, Michelle. After graduating valedictorian of her high school class in Hannibal, NY, she moved to Melbourne, FL to pursue her bachelor's degree. She received a Bachelor of Science (B.S.) degree in both molecular biology and marine biology at the Florida Institute of Technology in Melbourne, FL, in May, 2000. Under the mentorship of Dr. Rosalia Simmen, she received a Master of Science (M.S.) in animal sciences in August, 2002. After spending 2 years at the Moffitt Cancer and Research Institute at the University of South Florida, Jennelle returned to the University of Florida to complete her Ph.D. in animal sciences under the advisement of Dr. Sally Johnson. She is also the proud aunt and godmother of her nephew Brennen and has three cats, Merlin, Cali, and Barley.