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ABSTRACT

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Skeletal muscle regeneration provides a powerful model to study the cellular and molecular mechanism governing stem cell function since the whole process is mediated by a population of muscle-resident stem cells called satellite cells. The overall aim of this dissertation is to explore the roles of Notch signaling and a novel gene (Bex1) in satellite cell function and skeletal muscle regeneration.

The first part of this thesis is to examine how the Notch signaling pathway regulates the satellite cell self-renewal in a disease model. The MDX mouse is a well-established model for Duchenne muscular dystrophy (DMD) and characterized by progressive muscle degeneration and regeneration. Here, I showed that the number and activity of satellite cells in MDX mice were reduced in an age-dependent manner. Given that satellite cells can self-renew to maintain the homeostasis, I further demonstrated that the self-renewal capacity was defective in MDX mice. As the Notch signaling pathway has been reported to regulate satellite cell quiescence, I continued to confirmed that the self-renewal defect was due to the perturbed Notch in MDX satellite cells. Furthermore, I

attempted to genetically activate the Notch signaling pathway in satellite cells to improve muscle regeneration in MDX mice. Surprisingly, although the Notch activation increased the satellite cell number and rescued the self-renewal defects, it did not contribute to muscle regeneration. Nevertheless, this study extends our understanding of the Notch signaling pathway in the activity of satellite cells and in muscle regeneration.

The second part of this dissertation focuses on the roles of Bex1 in muscle regeneration. Bex1 is a novel gene with unknown functions in skeletal muscles. I first found that Bex1 was temporarily expressed in myogenic cells with a nuclear-cytoplasmic trafficking pattern during embryonic development and postnatal regeneration. The exclusive expression of Bex1 in differentiated myocytes suggests that Bex1 may play unknown roles in regulating myogenic differentiation. Previous studies suggested that Bex1 could regulate neuron cell cycle withdrawal. Here I demonstrated that Bex1 participated in myogenic differentiation independent of the cell cycle withdrawal. Instead, I showed that Bex1 promoted myoblast-myotube fusion *in vitro* and the regulation was independent of myogenic differentiation *per se*. However, Bex1 knockout mice appeared normal and did not exhibit obvious defects in the skeletal muscle. This study characterized the novel function of Bex1 in the myogenesis and facilitated the understanding of myoblast fusion process.

Collectively, the findings about how Notch signaling and Bex1 regulate the function of satellite cells as well as muscle regeneration derived from this dissertation will extend our understanding of the cellular and molecular

mechanism of muscle regeneration. Consequently, this dissertation can shed light on providing therapeutic avenues for the prevention and treatment of muscle diseases.

CHAPTER 1. INTRODUCTION

1.1 Skeletal Muscle Characteristics

Skeletal muscle constitutes around 50% of body mass and is the largest organ in our bodies (Huard et al., 2002). Under normal conditions, skeletal muscles have relatively uniform structure, composed of numbers of myofibers. Bundles of longitudinally aligned muscle fibers wrapped by extracellular matrix (ECM) are attached to either tendons or bones through myotendinous junctions, with blood vessels supplying nutrients and oxygen inside. Besides, adult muscle tissue contains a variety of interstitial and vessel-associated cells that display little mitotic activity under resting conditions. Different types of muscles have their unique structure and composition to fulfil their own specialized functions.

The classic functions of skeletal muscle are posture behavior, locomotive movement, and respiration. It is well established that skeletal muscles can contract and generate force through the “sliding mechanism” of myosin-rich thick filament over actin-rich thin filament upon neuronal stimulation (Huxley, 2000). Due to different requirements of muscle activities, the muscle fibers themselves are very heterogeneous in terms of structural and functional properties, referred as muscle fiber phenotype. Generally speaking, each individual skeletal muscle is a mixture of myofibers, ranging from a fast-contracting but easy-to-fatigue fiber

type to a slow-contracting but fatigue-resistant fiber type (**Table 1.1**). The overall contractile property of each individual skeletal muscle is dependent on the proportion of different fiber types. The regulatory mechanism of muscle fiber type diversity has been under intensive studies in the past few decades. Motor neuron has been demonstrated to play key roles in the remodeling process of muscle fiber type distribution (Calabria et al., 2009). The precise mechanism still remains elusive (Cavanaugh et al., 2012; Windisch et al., 1998). Taken together, the functional properties of skeletal muscles depend on a complex framework of muscle fibers, motor neuron, blood vessel and connective tissues.

Moreover, skeletal muscles play paramount roles in regulating the metabolism through uptaking glucose from the blood (Zurlo et al., 1990). Skeletal muscle metabolism is under hormonal control. Under fasting and resting conditions, blood glucose is mainly consumed by the brain and gut. In contrast, under the stimulus of insulin, skeletal muscles uptake 75% of blood glucose after a meal. Exercise training is another regulatory factor of skeletal muscle metabolism. For instance, endurance training increases the mitochondria content of skeletal muscles and up-regulates the insulin-sensitive glucose transporter, GLUT4 (Lessard et al., 2007; Ren et al., 1994). Loss of muscle mass under the circumstances of aging or degenerative myopathy can also lead to metabolic syndromes (Biolo et al., 2014). Due to the large volume of skeletal muscles, their metabolism affects the metabolic condition of the whole organism. As such, skeletal muscle is the primary site of insulin resistance under many metabolic

diseases conditions, including type 2 diabetes and obesity (DeFronzo and Tripathy, 2009).

1.2 Skeletal Muscle Development

In vertebrates, skeletal muscles are derived from paraxial mesoderm, which segregates into somites, including sclerotome and dermomyotome (Christ and Ordahl, 1995). The sclerotome, the ventral part of the somites, gives rise to the cartilage and bone while the dermomyotome, the dorsal part of the somites, contributes to the skeletal muscles of the body and limbs. During the embryogenesis, the muscle progenitor cells delaminate from dermomyotome and migrate to the limb bud, where they proliferate, commit to myogenic lineage and differentiate into skeletal muscle.

A number of transcriptional factors have been found to coordinately regulate the progression of myogenic lineage. Importantly, the embryonic progenitors can be characterized by Pax3/Pax7 expression. Then the myogenic commitment of muscle progenitor cells requires the up-regulation of two basic helix-loop-helix transcriptional factors, Myf5 and MyoD. These transcriptional factors belong to the muscle regulatory factor family (MRFs). In the absence of these two factors, those cells that originally develop to the muscles remain multipotent and adopt other cell fates, indicating the indispensable roles in muscle cell specification (Kablar et al., 1998; Rudnicki et al., 1993). In the presence of Myf5 alone, the initiation of myogenesis is delayed, suggesting that Myf5 itself can not sufficiently regulate the onset of myogenesis (Kablar et al., 1997). After the up-regulation of Myf5 and MyoD, cells proceed to the myoblast

stage. Then the myoblasts undergo extensive proliferation under the control of a number of regulatory factors, including Myf5 and MyoD. During the proliferation stage, some myoblasts withdraw from cell cycle and start to express some late-stage MRFs, myogenin and MRF4. The expression of myogenin and MRF4 qualifies a cell as a myocyte, referring to a terminally differentiated muscle cell. Both myogenin and MRF4 play essential roles in determining muscle differentiation program, as indicated by severe defects of muscle development in the myogenin and MRF4 knock-out mice. The myogenin knock-out mice die at the embryo stage with almost total loss of myofiber formation (Hasty et al., 1993; Nabeshima et al., 1993). The MRF4 knock-out mice exhibit different phenotypes, depending on which alleles were deleted (Patapoutian et al., 1995; Yoon et al., 1997; Zhang et al., 1995). Nevertheless, those phenotypes all indicate the role of *MRF4* in the late stage of myogenesis. With the myogenesis proceeding, the myocytes fuse to each other to form primary muscle fibers initially. Under the formation of innervations, another wave of myoblast fusion gives rise to the secondary muscle fibers. Subsequently, the muscle mass grows extensively during the fetal period and postnatally. During this muscle development process, it is noteworthy that some myoblast did not differentiate to myocyte but withdraw from cell cycle and back to quiescence. Those cells are located between basal laminin and muscle fiber membrane, defined as satellite cells. They are regarded as the adult muscle progenitor cells which can initiate the postnatal myogenesis (Anderson, 2006; Cheung and Rando, 2013; Kuang and Rudnicki, 2008; Wagers and Conboy, 2005).

1.3 Skeletal Muscle Regeneration

Under normal conditions, skeletal muscles are relatively stable with little turnover of myonuclei, no more than 1-2 percent per week (Schmalbruch and Lewis, 2000). However, skeletal muscles are very susceptible to injuries. In sports, muscle injuries are very common, covering 10-55 percent of all sustained injuries (Beiner and Jokl, 2001; Best and Hunter, 2000; Garrett, 1996; Huard et al., 2002). Skeletal muscle injuries can result from a variety of reasons, including contusion, strain, laceration, or a mixture of these reasons. As a matter of fact, more than 90 percent of muscle injuries are contusions and strains, which mostly lead to physical trauma without significant muscle loss (Beiner and Jokl, 2001; Counsel and Breidahl, 2010). Under these situations, skeletal muscles have a robust capacity to initiate a highly orchestrated regeneration process involving various cellular responses to prevent further muscle loss.

In spite of the types or severity of muscle injuries, muscle regeneration follows a stereotype, which can be generally divided into two phases listed as follows.

i. The decomposition and inflammatory phase characterized by the necrosis of muscle fibers and the activation of inflammatory responses.
ii. The repair and remodeling phase characterized by the phagocytosis of necrotic muscle fibers, generation of new muscle fibers by myogenic cells and formation of a tissue scar.

1.3.1 The Degeneration Phase

Muscle degeneration starts with the necrosis of damaged muscle fibers. Following muscle injury, the sarcolemma of muscle fibers are disrupted, leading to the increased muscle fiber permeability. Consequently, the intracellular proteins of muscle fibers can be released to the extracellular environment, such as creatine kinase. The expression of creatine kinase is usually restricted to the cytosol of muscle fibers. Therefore its elevated level in serum can indicate the occurrence of lesions of the muscle cell membrane (Coulton et al., 1988; Percy et al., 1979). Similarly, it was recently demonstrated that the serum levels of several muscle specific RNAs (myoMIRs), including mir1, mir206 and mir133, can also serve as biomarkers for muscle degeneration (Mizuno et al., 2011; Zaharieva et al., 2013). On the other hand, some extracellular substances may diffuse into the muscle fibers. Based on this assumption, various dyes have been developed to faithfully mark the degenerated fibers since they can be easily absorbed by damaged muscle fibers (Hamer et al., 2002; Palacio et al., 2002).

The most significant event due to the increased permeability is the influx of calcium or calcium release from sarcoplasmic reticulum. The loss of calcium homeostasis leads to activation of calcium-dependent proteolysis and promotes the rapid disintegration of myofibrils. For instance, calpains are calcium-activated proteases and play key roles in the decomposition of myofibril and cytoskeletal proteins (Belcastro et al., 1998).

During the early phase of muscle degeneration, the degradation of muscle fibers is accompanied with the infiltration of inflammatory cells. Previous studies

have demonstrated that the injured muscle fibers can release signals to activate the inflammatory cells residing in the muscles. Afterwards, those local inflammatory cells can continue to recruit leukocyte cells to the damaged sites via the chemotactic machinery. Specifically, neutrophils and macrophages are two major inflammatory cells in response to muscle injury (Arnold et al., 2007; Pizza et al., 2005).

Upon muscle damage, neutrophils migrate rapidly to the injury site within 6 hours, which evokes the local inflammation response. It is well established that neutrophils have a robust capacity to produce reactive oxygen species (ROS). Therefore as the neutrophils function, the dramatic increase of ROS levels speed up the disruption of muscle fiber structure (Fielding et al., 1993; Orimo et al., 1991). Subsequently, neutrophils are replaced by macrophages, the most predominant inflammatory cells invading injury sites. Basically, two distinct macrophages function in the muscle regeneration. At first, M1 macrophages characterized by the surface marker CD68+/CD163-, reach the concentration peak within 24 hours post onset of muscle injury and decrease rapidly afterwards. These M1 macrophages can secrete pro-inflammatory cytokines to promote inflammation and phagocytize cellular debris. Then M2 macrophage, characterized by the surface maker CD68-/CD163+, secretes anti-inflammatory cytokines, which sustain until the termination of the inflammation (Cantini et al., 2002; Lescaudron et al., 1999; Sonnet et al., 2006).

It is worth mentioning that both neutrophils and macrophages have significant roles in regulating the behavior of satellite cells. In detail, neutrophils

can activate satellite cells to the injury sites while macrophages can regulate the proliferation and differentiation of satellite cells (Merly et al., 1999; St Pierre Schneider et al., 2002), both of which prepare muscle tissue to proceed to the next stage, muscle repair phase.

1.3.2 The Repair Phase

Skeletal muscle regeneration has drawn intense attention for quite a long time in that muscle regeneration recapitulates embryonic myogenesis to some extent, which provides a powerful model to study muscle development. Long-standing histological characteristics are still being utilized to distinguish regenerating muscle fibers, such as the relatively smaller caliber and centrally located nuclei (Hall-Craggs and Seyan, 1975).

The muscle regeneration is a complex but highly synchronized process. The hallmark of this stage is represented by the extensive cell proliferation (Grounds et al., 2002; Hawke and Garry, 2001). During the regeneration process, various types of cells infiltrate in the injury sites, including satellite cells, inflammatory cells, blood vessel cells, neuron cells and so forth. Satellite cells migrate to the injury sites and differentiate to myoblasts. These myoblasts can undergo extensive proliferation to reach the sufficient cell number, which is extremely important for muscle regeneration, as indicated by the reduction of regeneration capacity after treated with proliferation inhibitors or irradiation (Quinlan et al., 1995; Wakeford et al., 1991; Weller et al., 1991). Those large quantities of myoblasts can either fuse to the existing muscle fibers for repair or

fuse with each other to form new muscle fibers (Darr and Schultz, 1987; Snow, 1978).

Of note, the myoblasts are not the only source of progenitor cells which are responsible for muscle regeneration. This raises intense interest and controversy concerning whether and how other muscle-derived stem cells and circulating progenitor cells can contribute to the muscle regeneration. To date, there are several types of cells isolated from skeletal muscles, which have been demonstrated to give rise to myogenic lineage cells, including bone marrow stem cells, muscle side population cells, PW1+ interstitial cells, mesoangioblasts, pericytes, and post-natal muscle derived stem cells, and CD133+ cells and so forth (Lee et al., 2000; Peault et al., 2007; Peng and Huard, 2004). Currently, it remains unknown how significantly these cells contribute to myogenic differentiation.

Among those different cell populations, post-natal muscle derived stem cells displays several advantages over other cell populations in cell transplantation treatment, including remarkable multipotency and long-term survival rate (Cao et al., 2003; Deasy et al., 2005; Lee et al., 2000). In addition, the origin of these post-natal muscle derived stem cells has been a topic of great interest. Emerging evidences show that those cells are from vascular endothelium (Tavian et al., 2005), suggesting that augmenting vascular supply to the wound area may be conducive to muscle regeneration.

1.4 Satellite Cells

Satellite cells were first observed around half a century ago (Mauro, 1961). These cells have been regarded as muscle stem cells in that satellite cells have been demonstrated to have the capability to replenish themselves and give rise to myogenic progeny (Bischoff, 1975; Collins et al., 2005; Konigsberg et al., 1975; Kuang et al., 2007; Lipton and Schultz, 1979; Montarras et al., 2005; Moss and Leblond, 1971; Sherwood et al., 2004). The identification of satellite cells raised them as the top candidate for post-natal muscle growth and regeneration. Indeed, they do play indispensable roles in muscle regeneration under a complex regulatory network, which has stimulated researchers to develop cell-based strategies to treat degenerative muscle diseases, such as Duchnne Muscular Dystrophy. However, the efficiency of these strategies is limited due to the low survival rate and migration capacity of the transplanted cells. Therefore it is imperative to make a more comprehensive understanding about satellite cell characteristics in order to manipulate quiescence, proliferation and differentiation of satellite cells.

1.4.1 Identification of Satellite Cells

Satellite cells can be identified based on a unique location with the perspective of anatomy. They are located between the basal lamina and the muscle fiber membrane along the whole fiber. Under the electron microscope, satellite cells with the “wedge” appearances exhibit condensed interphase chromatin, which is consistent with the notion that most satellite cells stay in the quiescent state and are transcriptionally inactive in the resting muscles (Mauro,

1961; Schultz et al., 1978). In addition, satellite cells can be identified in single fibers isolated from skeletal muscles through immuno-fluorescence imaging, which is dependent on specific biomarkers. In adult skeletal muscle, nearly every satellite cell expresses the paired box 7 (Pax7), which has been well-accepted as the canonical biomarker for satellite cells (Seale et al., 2000). Besides, a number of transcriptional factors have been utilized in identification of satellite cells, such as Pax3 and Myf5 (Buckingham et al., 2003; Cornelison and Wold, 1997). Also, some membrane proteins have been implicated in the identification of satellite cells, such as cell surface attachment receptor alpha7-intergin (Burkin and Kaufman, 1999; Gnocchi et al., 2009) and cell adhesion protein M-cadherin (Irintchev et al., 1994). It is noteworthy that some of these aforementioned markers can also be expressed in other types of cells. Therefore, it is necessary to identify satellite cells with combined fluorescence labeling.

1.4.2 Niche Regulation of Satellite Cells

In adult skeletal muscles, satellite cells are a major source for muscle regeneration. In resting conditions, satellite cells are mitotically inactive. Upon injury, satellite cells undergo a process of cytoplasmic volume expansion and heterochromatin reduction to become mitotically active. After satellite cells enter the cell cycle, they start to up-regulate the expression level of the basic helix-loop-helix transcriptional factors Myf5 and MyoD then progress to the highly proliferative myoblasts. During the proliferation, some cells withdraw from the cell cycle and start to express myogenin, subsequently differentiating to the new muscle fibers while the others lose their myogenic signature by down regulating

the expression level of MyoD and return to the quiescent state to replenish satellite cell pools (Bentzinger et al., 2012). During the whole process of muscle regeneration, the behaviors of satellite cells, including self-renewal, proliferation as well as differentiation, are under a precisely coordinated regulation by the surrounding cells which constitute the satellite cell niche.

In adult skeletal muscles, the total number of satellite cells remains relatively constant even with multiple rounds of muscle injury and repair. This homeostasis is largely attributable to the self-renewal capacity (Collins et al., 2005). The regulation of self-renewal is predominantly dependent on the extrinsic factor released by the surrounding cells residing in the satellite cell niche. Indeed, the cellular constituents of satellite cell niche are under a dynamic state. In particular, the components of quiescent satellite cells niche are significantly different from those of activated satellite cells niche. When satellite cells are quiescent, the satellite cell niche contains relatively less cell types, such as muscle fibers and vessel-associated cells.

Besides, this microenvironment remains static and exerts effects on maintenance of satellite cell quiescence through a variety of signaling pathways. Previous studies have corroborated that Notch signaling pathway plays key roles in the maintenance of satellite cell quiescence, wherein the genetic deletion of Rbpj, a main effecter of the Notch signaling pathway, activates satellite cells significantly (Bjornson et al., 2012; Mourikis et al., 2012). Considering the relative few cell types in the quiescent satellite cell niche, the Notch regulation may be derived from the muscle fibers within the satellite cell niche.

During cell proliferation, apart from a small proportion of satellite cells return to the quiescence, the majority of satellite cells remain active and further become more differentiated myogenic cells. This process is also under the regulation of circumambient cells in the niche. However, comparing to the quiescent niche, the niche of activated satellite cells becomes much more complicated with a spectrum of infiltrating cells.

First of all, immune cells play critical roles in regulating the behavior of activated satellite cells, also called myoblasts. In response to muscle injury, varieties of immune cells are quickly recruited to the wound sites and infiltrate to the niche. In particular, the predominant immune cells invading the injury sites are macrophages, basically with two subtypes, M1 and M2. M1 macrophages can promote myoblast proliferation while M2 can induce myoblast differentiation (Deng et al., 2012; Ruffell et al., 2009). This is supported by the observation that M1 macrophages accumulate close to the proliferating myoblast while M2 macrophages are proximate to the differentiating myogenic cells (Saclier et al., 2013).

Secondly, fibrogenic cells are also major contributors involved in the niche regulation. It is well agreed that the extracellular matrix (ECM) play key roles in regulating satellite cell behaviors. During muscle regeneration, ECM in the niche is largely and dynamically reorganized (Goetsch et al., 2003). In this process, fibrogenic cells including fibroblast and FAPs (fibro/adipogenic progenitors) can contribute to the deposition of several ECM components, such as different collagen isoforms, which participate in the niche regulation. It is noteworthy that

the FAPs and the myogenic cells can interplay with each other. FAPs can secrete IL6 to induce myogenic differentiation while the formation of myotube inhibits the differentiation of FAPs to adipocytes (Joe et al., 2010; Uezumi et al., 2010). In addition, the endothelial and periendothelial cells as well as the muscle lineage cells all play important roles in the niche regulation of activated myoblast (Abou-Khalil et al., 2010).

Due to the complicated constituents of the activated niche, the mechanisms that integrate different cell types are highly complex and further studies will unravel promising concepts in basic stem cell biology and also provide therapeutic avenues for muscular diseases.

1.4.3 Heterogeneity of Satellite Cells

Satellite cells were first considered as a homologous population of cells, which are committed muscle progenitor cells. As technology advances, emerging evidence has demonstrated that satellite cells are a heterogeneous group with respective to several characteristics, such as gene expression profiles, myogenic differentiation potential and intrinsic self-renewal capacity.

As for the gene expression profile of satellite cells, it is worth mentioning that the satellite cells do not always express those aforementioned satellite cell markers. For instance, examination of the aforementioned markers, CD34 and M-cadherin, revealed that a group of satellite cells are lack of those markers (Beauchamp et al., 2000). Recently, several novel biomarkers for satellite cells have been discovered with different expression patterns. The nuclear envelope proteins lamin A/C and emerin were universally found to be expressed in

quiescent and activated satellite cells whereas Jagged-1, a Notch ligand, was preferably expressed in activated satellite cells (Gnocchi et al., 2009). Therefore, the molecular signatures of satellite cells vary greatly. The combination of those biomarkers may have great implication in delineating the subpopulation of satellite cells and further interpreting the biological function of different subpopulations. However, the biological significance of this heterogeneity still needs to be clarified.

Satellite cells are very heterogeneous in their propensity of myogenic differentiation. It was observed that satellite cells from different types of muscles, including tibialis anterior (TA), extensor digitorum longus (EDL) and soleus, can contribute to the muscle regeneration differently after transplantation into MDX/nude mice (Collins et al., 2005). This result implies that the different sources of satellite cells are distinct inherently in the potential of myogenic proliferation/differentiation. It can be supported by another observation of varied levels of Myf5 in satellite cells, with Myf5 heterozygous cells more committed to myogenic differentiation compare to wild type control (Gayraud-Morel et al., 2012). This heterogeneity of satellite cells in myogenic differentiation may have a great advantage to effectively balance between satellite cells pool maintaining and myogenic differentiation requirement.

Another evidence of satellite cells heterogeneity comes from their intrinsic self-renewal activity. It has been corroborated that satellite cells are heterogeneous based on the expression level of Myf5, one of the earliest transcriptional factors for myogenic commitment (Kuang et al., 2007). In detail,

Myf5- satellite cells defined a 10% subpopulation while Myf5+ satellite cells represent the other 90%. It has been demonstrated that the Myf5- satellite cells can give rise to Myf5+ cells, indicating a hierarchical relationship between those two subpopulations. Cell transplantation experiments demonstrated that Myf5- satellite cells can not only undergo myogenic differentiation but also replenish satellite cell pools while Myf5+ satellite cells cannot self-renew but readily differentiate for muscle regeneration, which suggests that the self-renewal capacity resides in the Myf5- satellite cell subpopulation rather than Myf5+ cells. Altogether, these observations revealed the heterogeneity of self-renewal capacity residing in satellite cells. In this way, only a paucity of satellite cells can be regarded as true stem cells.

In summary, a variety of studies have substantiated that satellite cells are a very heterogeneous population. However, the biological significance of this heterogeneity remains poorly understood. Moreover, the intrinsic difference between these heterogeneous subpopulation at the molecular and functional levels needs to further clarified, which will facilitate in elucidating the mechanisms defining the different subpopulations as well as manipulating satellite cell behaviors in muscle regeneration.

1.4.4 Mechanisms of Satellite Cell Self-renewal

Due to the critical roles of self-renewal, the mechanism governing self-renewal has drawn intense attention in the field. In general, it was proposed that satellite cells can replenish themselves either through an asymmetrical division generating one quiescent daughter cell and the other differentiating daughter cell

or through a stochastic process wherein the progeny cells are the same at first but they take different cell fates afterwards.

Emerging studies have demonstrated that satellite cells undergo self-renewal through asymmetrical cell division. One of the evidence is based on the distribution of Numb, one cell fate determinant, in the daughter cells after cell division. It was observed that Numb is asymmetrically distributed in some but not all satellite cells (Conboy and Rando, 2002). In line with this observation, by BrdU labeled DNA strand segregation experiment, it was discovered that all “old” template DNA strands are co-segregated to the more stem-like daughter cells expressing the stem cell marker Sca1. Concomitantly, all “younger” template DNA strands are inherited by more differentiating daughter cells expressing the differentiation marker Desmin (Conboy et al., 2007). Based on the “immortal DNA strand” theory, the retention of old DNA strand can protect stem cells from accumulating mutations during DNA replication. Furthermore, it has been directly observed that the non-committed Pax7+Myf5- satellite cell can divide asymmetrically into two daughter cells with different orientation in the satellite cell niche, wherein one non-committed Pax7+Myf5- daughter cell is exposed to the basal lamina and the other committed Pax7+Myf5+ daughter cell is exposed to the host myofiber (Kuang et al., 2007).

In summary, through asymmetrical cell division, satellite cells can continuously replenish satellite cell pools in the course of muscle regeneration. This asymmetrical division pattern has been proposed to be dependent on the

different signals. But it remains unclear which signals play predominant roles and how these signals coordinate with each other to determine satellite cell fates.

1.5 Duchenne Muscular Dystrophy

During the contraction of muscle fibers, the fibers have a particular protein complex structure to buffer against mechanical stress. Genetic mutation of the elements of the complex can cause muscular dystrophy with different extents in term of onset age and dysfunctional muscles as well as severity, depending on how the mutated product affects the mechanosensory signaling network. Due to the genetic defects, muscular dystrophy is characterized by the progressive muscle weakness and degeneration. However, muscles have multifaceted repair system for regeneration. Upon injury, muscles can readily response to the stimuli and initiate a series of orchestrated events to fulfill the regeneration process. Thus, the pathophysiological hallmark of muscle dystrophy is repetitive muscle necrosis and regeneration. With the diseases advances, muscle degeneration gradually overtakes the regenerative capacity. Consequently, the muscle fibers are replaced by the fibrotic and adipose tissue, which leave muscle increasingly weak and eventually nonfunctional.

1.5.1 Characteristics of Duchenne Muscular Dystrophy

Duchenne Muscular Dystrophy (DMD) is the most severe muscular dystrophy, characterized by widespread muscle wasting, leading to loss of mobility, cardiovascular and respiratory failure, and finally death. DMD is an X-linked recessive muscular disorder with one of the highest mutation rates, affecting1 in 3500 male births (Blake et al., 2002). DMD patients are usually

bound to wheelchairs by 12 years age and died of respiratory failure in their early twenties. DMD is caused by mutations in the *Dystrophin* gene which is regarded as the largest in the human genome, containing 2.6 million bp DNA and 79 exons. It was estimated that around 60% mutations are the major frame-shift errors in the genome due to insertions and deletions while around 40% are due to point mutations and minor genome rearrangements (Hoffman and Dressman, 2001).

During muscle contraction, dystrophin can provide a structural link between the cytoskeleton and the extracullular matrix by binding to the actin with the amino-terminus as well as to the sarcolemma with the carboxyl-terminus through Dystrophin Associated Protein Complex (DAPC) (Brenman et al., 1996; Ibraghimov-Beskrovnaya et al., 1992; Rybakova et al., 2000). To form the complex, dytrophin first binds to the transmembrane β-dystroglycan together with α-dystroglycan, which constitutes the dystroglycan subcomplex. Also, four isoforms of sarcoglycans can complex with sarcospan to form the sarcoglycan subcomplex. Additionally, two cytoplasmic components, dystrobrevins and syntrophins, can be anchored to the carboxyl terminus of dystrophin. Of note, syntrophins can interact with nitric oxide synthase (NOS) (Adams et al., 2001; Ibraghimov-Beskrovnaya et al., 1992), which can regulate oxidative stress through NO production, and the recruitment of NOS to the sarcolemma is largely dependent on dystrophin (Brenman et al., 1995; Chang et al., 1996). In summary, DAPC has both structural/mechanical and signaling roles to maintain the integrity of sarcolemma.

1.5.2 Pathophysiology of Duchenne Muscular Dystrophy

In DMD, the absence of dystrophin renders the muscle membrane fragile to normal contraction. The disruption of membrane initiates a series of degeneration events as well as cellular protective responses. Currently, several pathophysiological perspectives have been investigated, including membrane fragility, impaired calcium homeostasis, and free radical damages. A comprehensive understanding of the pathophysiology underlying the deficits due to the absence of dystrophin will facilitate in developing new approaches for the treatment of Duchenne muscular dystrophy.

1.5.2.1 Membrane Fragility

Membrane fragility in DMD patients has been corroborated by multiple lines of evidence. It was demonstrated that myofibers in DMD patients accumulate various non-muscle proteins, indicative of membrane permeability. Consistent with this observation, several muscle-specific proteins and microRNAs, have been detected in the serum of DMD patients (Mizuno et al., 2011; Percy et al., 1979). These results can be supported by electron microscopy observation of membrane lesion in the DMD muscles (Mokri and Engel, 1975).

To make matters worse, the compromised integrity of the sarcolemma deteriorates under sustained exercise, especially eccentric contraction. Sustained exercises can cause lesion of myofiber membrane, which could be detected by the incorporation of Evans Blue, a dye developed for detection of muscle membrane permeability (Hamer et al., 2002). It was found that dystrophic muscles had tendency to incorporate more Evans Blue compared to WT controls

under sustained exercise (Straub et al., 1997). Notably, eccentric contraction represents the muscle contraction to the maximal length, which exerts significant mechanical stress on the sarcolemma. With the eccentric contraction, the ability of maintaining sarcolemma integrity in DMD patients is drastically reduced (Petrof et al., 1993). Therefore, the membrane fragility under mechanical stress contributes to the pathology of DMD, which suggests that physical therapy may be mandatory while excessive activity can be harmful to stabilize muscle condition in DMD patients.

1.5.2.2 Impaired Calcium Homeostasis

Calcium homeostasis is essential for a variety of muscle functions. Loss of calcium balance leads to dysfunctional muscles in various muscle diseases, including Duchenne muscular dystrophy. Indeed, impaired calcium homeostasis could be a signature event in the onset of muscular dystrophy as calcium influx was sufficient to induce dystrophic phenotype (Millay et al., 2009).

The approaches by which extracellular calcium enters the muscle cells have not been fully clarified. It was speculated that extracellular calcium enters muscle fibers simply through disrupted membrane. However, multiple lines of evidence demonstrated that extracellular calcium entered the dystrophic muscles by specific channels (Alderton and Steinhardt, 2000; Franco and Lansman, 1990; Matsumura et al., 2011; Yeung et al., 2005). These channels have been detected with altered expression levels in dystrophic muscles. More relevantly, over-expression of these channels could result in the cardiomyopathy, which further

suggests that these channels have pathological implication in muscular dystrophy (Iwata et al., 2003; Millay et al., 2009).

The calcium homeostasis was not promptly destroyed upon membrane disruption. Indeed, given that muscle fibers have strong capability to balance the calcium concentration, the calcium homeostasis can be maintained within a short period. With the influx advances, high amount of extracellular calcium inevitably subverts the muscle's capacity of maintaining calcium concentration and subsequently activates a number of calcium-dependent proteases. In particular, calpains can be activated by the calcium influx and play paramount roles in the degradation of muscle membrane protein, which further induces muscle necrosis. The pathophysiology of calcium influx has shed light on new strategy to improve muscle dystrophic phenotype. As such, some progress has been made to improve dystrophic phenotype by means of introducing the blockers of calcium channels and inhibitors of calcium-dependent proteases (Bonuccelli et al., 2003; Iwata et al., 2009; Yeung et al., 2005). Further attempts should be focused on investigating the detailed mechanism how calcium influx occurs during the onset of dystrophic phenotype.

1.5.2.3 Free Radical Damage

Free radical damage, also referred as oxidative stress, has been extensively investigated in various pathological conditions, including muscle wasting and muscular dystrophies (Arthur et al., 2008; Rando, 2002). It has been defined as the pathological condition wherein the endogenous antioxidants have been overweighed by the reactive oxidant species (ROS). It has been

determined that dystrophin-deficient myotubes are susceptible to oxidative damages (Rando et al., 1998), suggesting that oxidative stress may be involved in the pathophysiology of muscle dystrophy.

One of the most characterized molecules associated with oxidative stress is Nitric Oxide (NO), which can be generated by the neuronal nitric oxide synthase (nNOS). It has been shown that nNOS directly binds to the syntrophins, one component of DAPC, indicating that the collapse of DAPC in dystrophic muscles might lead to disturbance of NO generation. Indeed, the dissociation of DAPC results in the translocation of nNOS from plasma membrane to cytoplasm as well as a concomitant reduction of NO production. Conversely, ectopic expression of nNOS could dramatically rescue the dystrophic phenotype (Wehling et al., 2001). Paradoxically, NO plays dual roles in the process of oxidative stress, indicated by previous study showing that NO can function not only as a free radical reacting with superoxide but also as an antioxidant to relieve oxidative stress (Touboul et al., 2005). Therefore, the pathological implication of NO in muscular dystrophy is complicated and it needs further attempts for complete understanding and therapeutic manipulation. Nevertheless, introducing antioxidants to the dystrophic mice dramatically reduced the muscle degeneration (Buetler et al., 2002; Nakae et al., 2012). In summary, the free radical mediated muscle damage participates in the pathophysiology of muscle dystrophy, although the detailed mechanism needs further investigation.

1.5.3 Therapies of Duchenne Muscular Dystrophy

To date, there are no effective therapies for DMD, but a number of therapeutic strategies have been shown to delay or ameliorate the symptoms. Many of these therapies are merely effective to some extent in that they may aim at one aspect of the DMD pathogenesis. In particular, restoring the expression of dystrophin has been a promising trial for DMD therapy. It has been shown that as low as 30% restoration of dystrophin can prevent muscle weakness (Neri et al., 2007). However, due to the large amount of muscle mass including cardiac muscles and skeletal muscles, even 30% restoration has been a great challenge. Moreover, the restoration should be durable, which exposes another considerable hurdle to the therapy. Recently, significant progress has been made in seeking new strategies for DMD therapies, including gene therapies and cell therapies. Gene therapies can be performed by the traditional approach with direct protein replacement or the nascent methods involving manipulation of gene expression through modulation of transcription, post-transcriptional processing and translation. Cell therapies can be mediated by transplantation of stem cells bearing a functional *Dystrophin* gene, including autologous and allogenic transplantation depending on cell sources. The lessons from these therapies also shed light on the therapeutic treatment of other genetic disorders.

1.5.3.1 Dystrophin Restoration by Viral Delivery

Direct recovery of absent protein can be executed through viral gene delivery. In the past few decades, the genome information of virus and the mechanism of viral infection become transparent gradually, which significantly

relieves people's concern in the utilization of this viral therapy. As such, viral gene therapy has been applied in the treatment of multiple disorders due to its specific advantages in the efficiency of gene delivery.

Currently, vectors derived from adeno-associated virus (AAV) are the most promising vehicles for therapeutic gene delivery. It has been documented that AAV delivery proved effective and durable in some clinical trial (Vandenberghe and Auricchio, 2012). Still, this strategy faces significant challenges due to the immune response. Considering the large size of dystrophin, viral delivery of *Dystrophin* gene could probably evoke more intense immune response. Alternatively, several strategies using dystrophin fragment have been designed to include functional motifs to override the mutant carried in the patient, such as delivery of a functional fragment containing neuronal nitric oxide synthase (nNOS) binding sites (Lai et al., 2009).

Another limitation of viral gene therapy is to deliver dystrophin systemically. Local delivery of the *Dystrophin* gene under muscle specific promoters has been successful with no observed adverse effects (Wang et al., 2012). Due to the complexity of body system, systemic delivery requires further investigation on the vector design, particularly the promoter design. Also the durability after gene delivery is also a limiting factor of this therapy. To date, it remains unknown how long the dystrophin expression can be sustained after one delivery.

Taken together, viral gene delivery has been a promising approach but requires further studies to overcome immune responses essentially.

1.5.3.2 Dystrophin Restoration by Exon Skipping

Exon skipping holds promise in the treatment of muscular dystrophy. This strategy aims to correct the out-of-frame mutation by skipping the splicing of mutated exons and restoring the downstream frame, which could form a truncated but partially functional dystrophin protein. Recently, exon skipping has restored the dystrophin expression to a decent level in some clinical trials. Technically, different types of RNaseH dependent antisense oligonucleotides (AON) were designed to hybridize the sequences in or around the target exons to block the binding of splicing regulatory proteins and consequently skip the splicing of the mutated exon (Cirak et al., 2011; van Deutekom et al., 2007). To further optimize this strategy and enhance the efficiency of treatment, several bottleneck issues need to be emphasized.

First of all, the strategy is highly personalized and mutation specific, which means some mutated exons are easier to skip while the others are not. This is supported by the fact that some DMD patients can respond to the strategy effectively while some others have no responses (Mitrpant et al., 2009). One possible solution is to design multiple-exon skipping OANs, which has been confirmed to be feasible in principle (Aoki et al., 2012). Secondly, although AONs have been well tolerated with no safety issues at present (Kinali et al., 2009; van Deutekom et al., 2007), the long-term safety issues remain to be clarified, which is important especially for this life-long treatment. Next, the efficiency of AONs is restricted due to the poor cellular uptake, leading to variable levels of dystrophin restoration in skeletal and cardiac muscles.

Overall, although there are still several major obstacles in clinical trials, exon skipping has displayed promising therapeutic value in the DMD treatment.

1.5.3.3 Utrophin Compensation

Utrophin has been regarded as an autosomal homologue of dystrophin, and they share similar sequences and structures (Davies and Nowak, 2006). Moreover, utrophin can interact with members of DAPC and has the same function as dystrophin, which provides a bridge between cytoskeleton and extracellular matrix.

The major difference between utrophin and dystrophin is the expression pattern. In detail, dystrophin can be expressed along the whole muscle fibers while utrophin is restricted to the neuromuscular and myotendinous junctions. It has been observed that utrophin is increased and expanded beyond the neuromuscular and myotendinous junctions in dystrophic muscles, indicative a compensatory effect of dystrophin loss (Kleopa et al., 2006). This is also supported by the phenotype of dystrophin/utrophin double knockout mice, which have much worse phenotypes compared to dystrophin-deficient MDX mice (Deconinck et al., 1997).Furthermore, multiple studies have provided compelling evidence that the dystrophic phenotype can be ameliorated by increasing utrophin in muscles through a variety of approaches, including direct up-regulation of utrophin transcription, direct delivery of utrophin protein, and stabilization of endogeneous utrophin RNA or protein (Chakkalakal et al., 2008; Moorwood et al., 2013; Sonnemann et al., 2009; Tinsley et al., 2011).

In addition, utrophin has been observed to accumulate more in the slow oxidative muscle fibers comparing to the fast glycolytic muscle fibers. This has great implication since the slow oxidative muscle fibers can be more resistant to the dystrophic pathology in DMD patients (Webster et al., 1988). Therefore, promotion of slow oxidative myogenic program may be beneficial to the treatment of DMD. Activation of the calcineurin-NFAT (nuclear factor of activated T cells) or PGC1-α (peroxisome proliferator activated receptor γ coactivator 1 α) has been determined to promote slow oxidative program and increase the expression level of utrophin, which significantly improved dystrophic phenotype (Chakkalakal et al., 2004; Ljubicic et al., 2014; Selsby et al., 2012).

Compared to other gene therapies, utrophin compensation holds more significant therapeutic promise in that this strategy of increasing utrophin is effective irrespective of the dystrophin mutations and it can circumvent the hurdle of immune response in the dystrophin-based therapies.

1.5.3.4 Stem Cell Mediated Therapies

Cell therapies have gained intense interest for muscular dystrophy treatment and were carried out in the clinical trials earlier in the 1990s (Gussoni et al., 1999; Mendell et al., 1995).The strategy of cell therapies is to transplant cells bearing the functional *Dystrophin* gene to the dystrophic muscles. Theoretically, those transplanted cells could not only migrate to the injury sites for regeneration but also inhabit in the satellite cell niche to self-renew for future use. To date, multiple types of cells have been utilized for dystrophy therapies.

The first attempt of DMD cell therapy was to transplant myoblasts to the dystrophic muscles thanks to the ease of myoblast culture. This was first examined in MDX mice, and the results showed that transplanted myoblasts fused to MDX myofibers and relayed the expression of dystrophin in the dystrophic muscles (Partridge et al., 1989). However, myoblast transplantation in clinical trials failed to provide decent increase of dystrophin in DMD patients (Miller et al., 1997), which may be due to the low survival rate and inability to migrate. To improve this, multiple injections of large quantities of myoblasts were performed, leading to around 10% dystrophin production in the injury sites (Skuk et al., 2007). Still, the low efficiency of transplantation elicits researchers to seek other choices for cell therapies.

Compared with myoblasts, satellite cells point to a promising candidate for cell therapy of muscular dystrophy in that they could enter the satellite cell niche to self-renew and provide a long-time treatment. Satellite cells could be purified based on the surface markers and transplanted to MDX mice. After transplantation, those satellite cells contributed to 97% fibers and significantly improved muscle performance (Cerletti et al., 2008), yet several hurdles of satellite cell mediated therapy still need to be overcome. One of them is the inability to migrate over long distances, which restricted satellite cell therapy to local injection. Another challenge may come from the lower myogenic potential of satellite cells, which consequently reduced the efficiency of satellite cell therapy. A promising direction is to transplant cells with the matrix, such as myofibers, which have opened new fields in tissue engineering.

In addition to myoblasts and satellite cells, several other types of cells also have been studied for cell therapies of muscular dystrophy, such as bone marrow derived stem cells (Ferrari et al., 1998) and mesoangioblasts (Sampaolesi et al., 2006). Compared to myoblasts and satellite cells, these non-myogenic cells have advantages in the systematic delivery as they could be delivered to muscles through circulatory system, yet the main challenge how to increase the myogenic potential remains to be explored.

1.5.4 Animal Models of Duchenne Muscular Dystrophy

1.5.4.1 Mouse Models

To date, several animal models have been developed to study the fundamental principles and test the therapeutic approaches for DMD. The MDX mouse is the most widely used homologous animal model. The MDX mouse features a premature stop codon mutation in exon 23 of the *Dystrophin* gene, leading to little or no dystrophin protein production . The MDX mouse was born normal until the third postnatal week when the widespread muscle necrosis was initiated due to the absence of dystrophin. The muscle degeneration is followed by extensive muscle regeneration, indicated by centrally nucleated muscle fibers persisting through life. The MDX mouse has been a valuable tool for DMD study due to the genetic background and histological features.

However, there have been also quantities of debates on the value of the MDX mouse as a DMD model. The reason why so many debates arise is that the pathology of MDX is far more benign than that of DMD patients. In detail, the lifespan of MDX mouse is similar compared to the wild type mouse. The

cardiomyopathy and fibrosis, typical characteristics of severe dystrophic phenotype, do not occur until MDX mouse undergoes aging. This drawback has significantly limited the utilization of MDX mouse and impeded the translation to the human clinical trial of data gathered from MDX. To date, great efforts have been dedicated to improve the application of the MDX mouse by virtue of breeding it to the mouse with other genetic background.

The first attempting of breeding MDX with other genetic mouse was performed on the nude mouse when scientists aimed to reduce the immune responses during myogenic transplantation. Although this breeding did decrease the immune rejection as expected, the nude background had predominant effects on the disposition of collagen, one of the major components in the formation of fibrotic tissues, which raised problems in the analysis of the pathogenesis of muscle dystrophy (Morrison et al., 2005).

The second attempt was based on the discovery of utrophin, which is up-regulated in the MDX mouse and can compensate for the absence of dystrophin. This prompted researchers to generate the dytrophin and utrophin double knock-out mouse. Strikingly, the mouse null for the dystrophin and utrophin exhibits much more severe pathology compared to MDX, closely resembling the phenotypes in DMD patients, including decreased growth, reduced mobility, muscle weakness, spinal deformities, cardiomyopathy, myofibrosis and short lifespan. Therefore, dystrophin and utrophin double knockout mouse has been proposed to provide new insight into pathogenesis and an effective model to test therapy for the devastating disease (Deconinck et al., 1997).

Another attempt came from breeding MDX with telomerase null mouse according to the hypothesis that the much longer telomere of mouse compared to that of human confers almost infinite proliferative capacity to satellite cells, which can fuel muscle regeneration through whole life in mouse. The reduced proliferative potential of muscle stem cells in the double knockout mice led to more severe phenotype comparing to MDX alone. Thus, the milder phenotype in MDX compared to DMD patients could be partially attributable to longer telomeres. This double mutant mouse may prove useful for explaining the pathophysiology of DMD and testing the therapeutic intervention for DMD treatment (Sacco et al., 2010).

Altogether, these modifications to the MDX genetic background add more biological complexity during the translation of data obtained from mouse to human, indicating more caution need to be taken in the application.

1.5.4.2 Dog Models

Another important animal model for DMD is the Golden Retriever dog model. In this model, there is a splice-site mutation located in exon 7 of the *Dystrophin* gene, which causes the splicing of exon 7 from dystrophin transcript. As a consequence, the deletion of exon 7 shifts the open reading frame and leads to no protein production.

The dystrophic phenotype in this model is so severe that the mutant dogs die prematurely (Sharp et al., 1992). This model has been utilized primarily as a reference species to test therapeutic intervention before applied into clinical trial. However, one problem regarding Golden Retriever dystrophic dogs is that the

low survival rate makes it not only time-consuming but also very expensive to generate enough numbers for practical application. Besides, there are also several distinct phenotypes of muscle dystrophy between Golden Retriever dystrophic dogs and DMD patients due to the differences between these two species. For example, some Golden Retriever dystrophic dogs died soon after birth due to severe phenotype, which is very rare in DMD patients. Still, this emphasized that the species difference need to be taken into consideration when applying data gathered from animal model to human beings.

1.6 Signaling Pathways in Postnatal Myogenesis

Muscle regeneration involves a series of orchestrated events that require extensive communication of multiple signaling pathways and transcriptional factors during the satellite cell activation and myoblast proliferation/differentiation as well as myotube formation. To name a few, two classical developmental signaling pathway, Wnt and Notch, has been corroborated to play pivotal roles in the myogenic response during muscle regeneration.

1.6.1 Wnt Signaling Pathway

The canonical Wnt activation requires the binding of extracellular Wnt family glycoproteins with Frizzled receptors and LPR (low-density lipoprotein receptor-related protein). This interaction can promote the phosphorylation of Disheveled, which can inhibit GSK3-β activity (GSK3-β phosphorylated at tyrosine 206) (Yuan et al., 1999). In the cytoplasm, GSK3-β and β-catenin can form the complex together with Axin and adenomatous polyposis coli (APC), in which the β–catenin can be phosphorylated by GSK3-β and further degraded by

proteasome. The inhibition of GSK3-β by phosphorylated Disheveled stabilizes β–catenin and allows β–catenin to enter the nucleus and interact with TCF/LEF transcriptional factors to regulate expression of target genes, including *MyoD* and *myogenin* (Church and Francis-West, 2002; Ridgeway et al., 2000) (**Figure 1.1**).

Wnt signaling pathway regulates a series of biological processes, including cell morphology, cell adhesion, cell proliferation and cell fate determination. Because all these processes are highly active in skeletal muscle regeneration, it is plausible to assume that Wnt signaling pathway plays a key role in muscle repair. Indeed, mRNA transcripts of Wnt5a, 5b, 7a, and 7b are elevated at 4 days post cardiotoxin-induced injury (Polesskaya et al., 2003). Emerging evidences have shown how Wnt is involved in the postnatal myogenesis during muscle regeneration. However, the results were controversial between different research groups. Rando group determined that Wnt can promote myogenic differentiation only in the late stage of myogenesis (Brack et al., 2008). In contrast, Zammit group suggested that Wnt can promote self-renewal and inhibit myogenic differentiation by over-expression and knockdown of β-catenin in single fiber culture (Perez-Ruiz et al., 2008). Taken together, Wnt signaling pathway does have regulatory effects on postnatal myogenesis during muscle regeneration. However, further studies should be conducted to investigate the detailed mechanism.

1.6.2 Notch Signaling Pathway

Activation of Notch signaling pathway require the binding of ligands (Dll1, 3 and Jag1, 2 in mice) to Notch transmembrane receptors (Notch1-4 in mice).

Consequently, the binding induces the enzymatic cleavage of Notch receptors by metalloproteases and γ-secretases and then release the active truncated form of Notch receptors, named as Notch intracellular domain (NICD). Subsequently, NICD translocates from the cytoplasma to the nucleus, wherein NICD can interact with the CSL transcriptional repressors (CBF1/RBP-J, Suppressor of Hairless, and Lag-1) and convert them to transcriptional activators. The complex of NICD and CSL activates the expression of target genes, including *Hes* and *Hey* family genes (Artavanis-Tsakonas et al., 1999) (**Figure 1.2**).

During the myogenesis, Notch signaling pathway plays critical roles in mediating the communication between cells due to their transmembrane location. Notch receptors including Notch 1, Notch 2 and Notch 3 are all expressed abundantly in satellite cells (Kitamoto and Hanaoka, 2010). Upon muscle injury, the expression levels of the Notch 1 receptor together with the Notch ligand Delta 1 are both elevated on satellite cells and their neighbor muscle fibers (Conboy and Rando, 2002), which promotes the proliferation of satellite cells and inhibit the myogenic terminal differentiation. Inhibition of Notch by Notch antagonist or γ-secretase inhibitors leads to decreased proliferation and premature myogenic differentiation. Due to the lack of myoblast required for muscle regeneration, the inhibition of Notch deteriorates muscle repair (Kitzmann et al., 2006). Reversely, activated NICD can directly regulate *Pax7* to promote myoblast proliferation and inhibit myogenic differentiation. As a consequence, constitutive activation of Notch signaling pathway also spoils muscle regeneration (Wen et al., 2012). Based on the active participation in muscle regeneration, Notch has provided

promising avenues for the treatment of muscle diseases. However, extreme caution should be given to balance its function in myoblast proliferation and differentiation.

1.6.3 Interplay between Wnt and Notch Signaling Pathways

It is worthy to mention that Wnt and Notch can interplay with each other during muscle regeneration. In the early stage of myogenesis, Notch can promote myoblast proliferation and inhibit precocious differentiation. As myogenesis proceeds, Notch inactivation is necessary for myogenic terminal differentiation. It has been demonstrated that the activation of Wnt pathway plays antagonizing effects to Notch to facilitate the terminal differentiation. This is supported that inactivation of Notch signaling pathway is related to the dephosphorylation of GSK3-β at the tyrosine 216 site, which is important for the stabilization of β–catenin and Wnt activation (Conboy and Rando, 2002; Hagen et al., 2002). Consequently, at day one post muscle injury, when myoblasts proliferate extensively, Notch activity is high and Wnt activity is weak. While at day four post injury, myoblasts are ready to enter the terminal differentiation phase, in which Notch activity decreases and Wnt activity increases (Brack et al., 2008). Of note, it has been well documented that GSK3-β can directly bind to the NICD and regulate Notch target gene expression (Espinosa et al., 2003; Foltz et al., 2002). Thus it appears plausible to conclude that GSK3-β function as a bridge to connect Wnt and Notch signaling pathways. Therapeutic intervention of the balance between Wnt and Notch pathways through GSK3-β would be beneficial for cell fate decision during muscle regeneration.

1.7 Brain Expressed X-linked Protein 1 (Bex1)

1.7.1 Brain Expressed X-linked Gene Family

Bex1 belongs to the Bex gene family, which is relatively small but may play essential roles in the development and function in mammals. It was reported that Bex family was highly expressed at rat embryonic day 10 of development during ventral mesencephalon development, suggesting the role of Bex family in the dopamine neuron differentiation (Alvarez et al., 2005). To date, six members of the Bex family have been discovered, including Bex1Bex1Bex1*, Bex2, Bex3, Bex4, Bex5* and *Bex6*. Except *Bex6*, which is in chromosome 16, the other five members are located in the X chromosome with tandemly arrangement. In detail, *Bex5,* Bex1Bex1Bex1 and *Bex2* are located in the negative strand while *Bex3* and *Bex4* in the positive strand tandemly (Zhang, 2008). In addition, the gene structures of the whole family are well conserved. All of them contain three exons while the first two exons are not translated.

All the six members of Bex family have been characterized in term of their sequences and expression pattern in human, mice, and rats (**Figure 1.3**). For instance, the amino acid sequences of human Bex1 and Bex2 are almost identical (85% identity) and both of them are highly expressed in central nervous system, including pituitary, cerebellum, and temporal lobe (Alvarez et al., 2005). Bex3, also named as pHGR74 or NADE, has been determined to interact with low-affinity neurotrophin receptor (p75NTR) to mediate apoptosis signaling (Mukai et al., 2000). Also, it has been demonstrated that Bex3 is abundantly expressed in the human ovarian granulose cells (Rapp et al., 1990). Although

Bex3 is only 30% identical to Bex1 or Bex2 in their sequences, they still share some major function domains, indicating they may be involved in similar biological process. Indeed, Bex1, Bex2 and Bex3 are all expressed in the olfactory system (Behrens et al., 2003). In addition, Bex4 and Bex5 share 54% and 56% sequence identity with Bex3, respectively. And both of them are very similar in sequences and are highly expressed in brain. Besides, Bex4 is highly expressed in heart, skeletal muscle and liver, which may indicate that it is associated with high energy consumption. Compared to Bex4, Bex5 is more widely expressed (Alvarez et al., 2005). As a novel member of Bex family, Bex6 is the only one which is not expressed in human. Also Bex6 is not located in X chromosome but chromosome 16 in mouse, which suggests a distant relationship with other members. As for the subcellular localization, the six members of Bex family vary greatly. Bex1 is more confined to the nucleus while Bex3, Bex5 and Bex6 are expressed in cytoplasm. Bex2 and Bex4 can be expressed in both nucleus and cytoplasm (Alvarez et al., 2005). Taken together, the six members of Bex family are highly homologous but different in the expression pattern as well as the subcellular localization.

1.7.2 Bex1 Structure and Expression Pattern

Bex1 gene is located in the 53.77cM on X chromosome in mouse, which corresponds to the Xq22 region on human X chromosome. In mouse, the gene consists of three exons which span around 1.5kb of genomic DNA and the size of mRNA transcript is around 800bp. There are more transcripts expressed from the

maternally inherited allele compared with the paternally inherited allele, indicating an imprinted expression pattern (Brown and Kay, 1999). Bex1 protein has 128 amino acids, including a nuclear localization signal, a histidine-rich sequence close to the carboxyl terminal and a carboxyl terminal CLMP motif (Behrens et al., 2003; Brown and Kay, 1999).

In mouse, Bex1 is highly expressed in brain with low levels in lung and gonads (Brown and Kay, 1999). While in human, northern blot analysis revealed that the approximately 1kb mRNA transcript is expressed abundantly in brain, pancreas, ovary and testis while at low levels in heart, placenta, liver, kidney, thymus, spleen, prostate, small intestine, adrenal gland, thyroid, and spinal cord. There is no expression in lung, stomach, bone marrow, and skeletal muscles (Yang et al., 2002). Also, Bex1 was identified with an up-regulation after fertilization (Williams et al., 2002), indicating that Bex1 may play roles in embryonic development. Altogether, although the expression pattern was well characterized, the detailed function of Bex1 remains to be clarified, especially in some specific tissues.

1.7.3 Bex1 Functions

Bex1 was first identified with reduced expression in the teratocarcinoma cell line after treatment of retinoic acid and characterized in the analysis of imprinted genes between parthenogenetic and normal blastocysts (Brown and Kay, 1999; Faria et al., 1998). Multiple studies have been performed afterwards to explore the function of Bex1 in many biological processes. Bex family members are well conserved and the amino acid sequence of Bex1 has 87%

identity with Bex2 but lower similarity to other members (30% identity with Bex3, 25% identity with Bex4, 20% identity with Bex5, 18% identity with Bex6). Notably, some functional domains among the Bex family members are well conserved, suggesting that these Bex proteins may share the same implication in term of biological activity. For instance, researchers first found that Bex3 could bind p75NTR directly to mediate the neurotrophin signaling pathway (Mukai et al., 2000). Due to the similarity of sequences implicated in the p75NTR binding between Bex1 and Bex3, it has been proposed and finally confirmed that Bex1 can also bind to p75NTR to play roles in neurotrophin signaling pathway (Vilar et al., 2006). To date, the functions of Bex1 has been associated with tumorigenesis, p75NTR mediated cell cycle regulation, neuron regeneration, and muscle regeneration. However, the molecular mechanism behind Bex1 regulation has remained to be clarified.

1.7.3.1 Bex1 is A Tumor Suppressor.

To date, most Bex1-related studies focus on its contribution in the formation of various tumors, including pediatric intracranial ependymoma (Karakoula et al., 2014), oral squamous cell carcinoma (Lee et al., 2013), salivary gland adenoid cystic carcinoma (Shao et al., 2011), malignant glioma (Foltz et al., 2006) and so forth. Based on these studies, Bex1 has been determined as a tumor suppressor. Inactivation of tumor suppressor genes, together with overexpression of oncogenes, has been regarded as the dominant driving force of tumorigenesis. Inactivation of tumor suppressor genes can be attributable to multiple reasons. Epigenetic regulation is one of the reasons and has drawn

intense interest in the field in the past decades. The most common epigenetic mechanism implicated in the inactivation of tumor suppressor genes includes promoter hypermethylation and histone deacetylation. In those aforementioned Bex1-related tumors, Bex1 was markedly silenced in the tumor specimens by extensive promoter hypermethylation. Furthermore, Bex1 over-expression can dramatically inhibit cell proliferation and colony formation.

Besides its role of tumor suppressor in cancer, Bex1 is also essential to understanding the acquisition of chemoresistance in the progression of multiple tumors, such as breast cancer and leukemic cells. Chemotherapy can induce cell apoptosis and has been applied to the treatment of various cancers. Although an expanding amount of chemotherapy reagents have been developed in the past decades, chemoresistance of tumor cells still poses a significant challenge in cancer treatment. The mechanism of chemoresistance has been under intense investigation. Bex1 down-regulation in cancer cells is a novel mechanism by which cancer cells can obtain the chemoresistance and escaped apoptosis. In contrast, ectopic expression of Bex1 can sensitize cell to apoptosis induced by chemotherapy (Foltz et al., 2006). It has been recently demonstrated that Bex1 can promote imatinib-induced apoptosis through interacting with BCL-2 and suppressing the formation of anti-apoptotic complex Bcl-2/BAX (Xiao et al., 2014). Taken together, Bex1 functions as a tumor suppressor and can be a potential target for cancer therapy.

1.7.3.2 Bex1 Regulates Cell Cycle through p75NTR.

Understanding the involvement of Bex1 in tumor progression can be associated with the function of Bex1 in cell cycles. It has been found that Bex1 can prevent normal neuron cell cycling and inhibition of Bex1 in neuron cells can lead to sustained cell proliferation under the condition of growth arrest. Further studies have confirmed that Bex1 regulates cell cycle by interacting with p75NTR to participate in neurotrophin signaling pathway (Vilar et al., 2006).

Neurotrophin signaling pathway has been characterized to regulate cell survival, growth and differentiation in a variety of neuronal subpopulations. Two types of receptors are involved in the neurotrophin signaling pathway. One is the high affinity receptors (Trk tyrosine kinases) while the other is the low affinity receptors (the p75 neurotrophin receptor, p75NTR). It has been determined that two Bex family members, Bex1 and Bex3, can interact with p75NTR to initiate signal transduction (Mukai et al., 2000; Vilar et al., 2006).The binding of neurotrophin to p75NTR can lead to multiple physiological consequences in the neuron system, varying from cell apoptosis to neurite survival and even outgrowth. This is supported by the observation that p75NTR knockout mice have markedly less sensory neurons but normal motor neurons in dorsal root ganglia as well as dramatically increased cholinergic neurons in basal forebrain (Niklison-Chirou et al., 2013). These multifaceted functions of p75NTR signaling pathway reflects that other factors may be involved in the regulation process.

Furthermore, due to lack of intrinsic catalytic ability of p75NTR receptor, the signaling pathway is largely dependent on the p75NTR-interacting molecules.

Because of these different mediators, p75NTR can exert its multiple effects on cellular activity. For example, p75NTR can inhibit cell mitosis through interacting with SC1 and promote neurite outgrowth through activating RhoA as well as induce cell death through NRAGE (Chittka et al., 2004; Salehi et al., 2000; Taylor et al., 2001). However, the detailed mechanism how the interaction between p75NTR and interactors with or without catalytic capability mediates downstream signaling pathway and cellular response needs to be investigated specifically based on cellular context.

Vilar et al. has demonstrated that Bex1 and p75NTR share essentially indistinguishable expression pattern in the nervous system as well as vascular and mesenchymal structures during embryonic development. They continued to confirm that Bex1 can directly interact with p75NTR intracellular domain (Vilar et al., 2006). Considering the regulation of p75NTR signaling on the cell cycle and cell apoptosis, Bex1 expression levels may have great implication in the intracellular machinery controlling cell survival, proliferation and differentiation.

1.7.3.3 Bex1 Regulates Neuron Regeneration.

Regeneration in neuron system could be complicated partially due to the different regeneration capacity based on different location. In detail, axons in peripheral neuron system can be repaired after injury while axons in central neuron system do not preserve any regeneration capability. This difference can be attributable to both intrinsic and extrinsic factors. Axons in central neuron system are intrinsically lack of ability of regrowth. Besides, a variety of extrinsic factors in the central neuron system can be released to inhibit the regrowth, such

as myelin-associated glycoprotein (MAG) (McKerracher et al., 1994; Richardson et al., 1980). In contrast, the injured axons in peripheral neuron system can not only induce the intrinsic capacity of neuron regrowth but also stimulate multiple extrinsic regeneration-related gene expression to overcome the blockage of inhibitory factors .

Bex1 has been identified as one of the extrinsic regeneration genes, which is indicated by the observation that the expression level of Bex1 was elevated after axon injury as well as that Bex1 expression is consistent with several established regeneration genes in multiple mouse models. Also Bex1 knockout mice are deficient in axon regeneration after sciatic-nerve injury, a well-established model for studying neuron regeneration. Furthermore, Bex1 can counteract the inhibition of inhibitory factors during axon regeneration in that the motor neuron expressing Bex1 can grow longer axon than wild type in the presence of MAG. Therefore, Bex1 can be categorized as a regeneration gene during peripheral neuron repair (Khazaei et al., 2010).

To date, the mechanism of Bex1 involvement in the axon regeneration at the molecular level remains elusive. It was hypothesized that the interaction between Bex1 and p75NTR signaling pathway may be conducive to understanding the mechanism as p75NTR signaling pathway can regulate neuron cell growth and differentiation as well as p75NTR also interacts with MAG (Dubreuil et al., 2003; Robak et al., 2009; Vilar et al., 2006). Future endeavors need to be attempted to investigate the role of Bex1 in the neuron regeneration.

1.7.3.4 Bex1 Regulates Muscle Regeneration.

The relevance of Bex1 in muscle regeneration was discovered in a microarray aimed to identify a panel of genes with altered expression in CTX induced muscle injury, a well-established model for muscle regeneration (Yan et al., 2003). Consistently, the expression level of Bex1 was dysregulated in MDX mice (Turk et al., 2005), which undergo progressive muscle degeneration and regeneration. Bex1 knockout mice have prolonged proliferation and delayed differentiation kinetics. In addition, both cell cycle inhibitor genes and myogenic differentiation marker *myogenin* were down-regulated compared to regenerating wild type muscles (Koo et al., 2007).

It has been suggested that Bex1 regulates muscle regeneration through interacting with calmodulin (CaM) (Koo et al., 2007). And Bex1 and CaM co-localize in myogenic cells during muscle regeneration (Koo, 2010). CaM can bind calcium to activate calcium dependent kinases and initiate calcium signaling pathway. Due to the indispensable role of calcium in myogenesis, it is logical to hypothesize that the interaction between Bex1 and CaM contributes to muscle regeneration. Moreover, one can speculate that the interaction between Bex1 and p75NTR also contributes to the regeneration of skeletal muscles since p75NTR is also highly expressed in myogenic cells (Lomen-Hoerth and Shooter, 1995; Yamamoto et al., 1996). Besides, given that muscle regeneration is a complicated process which requires a series of coordinated responses from surrounding cells, this hypothesis is also supported by the observation that neurotrophins are secreted by various cells in the developing limb bud

(Henderson et al., 1993; Ip et al., 2001). Furthermore, analysis of their expression pattern during embryonic development shows that Bex1 and p75NTR can be concomitantly expressed in the somatic mesenchyme and heart (Cotrina et al., 2000; von Schack et al., 2001), suggesting that Bex1 could interact with neurotrophin signaling pathway in muscle regeneration. However, detailed mechanism underlying Bex1's function in satellite cells and muscle regeneration remains to be clarified.

1.8 Rationale and Objectives

Under normal conditions, skeletal muscles are very stable with little myonuclei turnover. However, skeletal muscles are susceptible to injury, which may lead to mass loss and functional deficiency. Skeletal muscles have a remarkable capacity to initiate the repairing process, muscle regeneration, to prevent further muscle loss and maintain normal muscle function. The overall aim of this dissertation is to study muscle regeneration process at the cellular and molecular level. In detail, two projects are included in this dissertation. The first one is to investigate how Notch signaling pathway regulates satellite cells behavior and further affects skeletal muscle regeneration in MDX mice. The second one is to explore the role of Bex1 in the myogenesis as well as muscle regeneration. This Chapter mainly reviews the current knowledge related to these two objectives. Satellite cells, which play an essential role in muscle regeneration, are emphasized. Importantly, it is worthy reviewing the knowledge related to MDX, the widely used mouse model for Duchenne muscle dystrophy, which is characterized by progressive muscle degeneration and regeneration.

Besides, a number of signaling pathways and transcriptional factors have been demonstrated to regulate muscle regeneration. In particular, Notch signaling pathway and Bex1 are discussed in more details to provide a comprehensive understanding in the follow-up experiments.

Muscle regeneration is a perfect model to study skeletal muscle stem cell biology in that it can facilitate exploring not only basic mechanisms of muscle development but also the therapeutic potentials of muscle stem cells. Therefore, unraveling the regulatory network behind muscle regeneration has drawn intense research attention in the field. To date, great progress has been made in understanding muscle regeneration, yet there are many unanswered question and unexplored areas. With this notion in mind, the roles of Notch signaling pathway and Bex1 gene in muscle regeneration were investigated in this dissertation. Notch signaling pathway plays multifaceted roles in the myogenesis. In particular, Notch can promote the self-renewal capacity of satellite cells, the predominant cellular source for muscle regeneration. Whether promoting satellite cell self-renewal contributes to muscle regeneration is an intriguing question. If so, enhancing satellite cell self-renewal may lead to improved muscle function in various degenerative and congenital muscle diseases, such as Duchenne muscular dystrophy. Next, Bex1 has been implicated in regeneration of motor neuron and skeletal muscles. However, the role of Bex1 in myogenic satellite cells and the underlying mechanisms remain to be elucidated. Several findings related to the function of other Bex family members may open new perspectives

in understanding the molecular mechanism of Bex1 regulation in muscle regeneration.

Table 1 1 Characteristics of Different Types of Muscle Fibers

Characteristics	Fiber Types	
	Slow	Fast
Contraction	Slow	Fast
Resistance to fatigue	High	Low
Generation of ATP	Aerobic	Anaerobic
Amount of Mitochondria	High	Low
Oxidative Capacity	High	Low
Glycolytic Capacity	Low	High

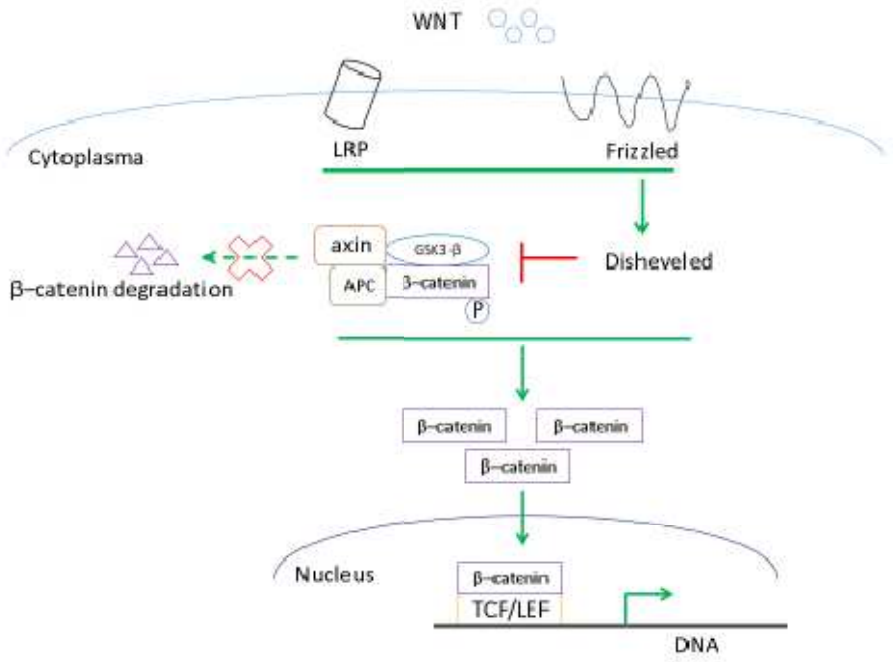

Figure 1.1 A Diagram of the Canonical Wnt Signaling Pathway
In the absence of Wnt ligands, β-catenin can interact with GSK3-β, Axin and APC and form a multi-protein complex, in which β-catenin can be phosphorylated and further degraded by the proteasome. Without β-catenin translocation to the nucleus, TCF/LEF transcriptional factors will be bound by the repressors, which blocks the gene transcription. While the binding of Wnt ligands to the receptors can activate Disheveled, which blocks the multi-protein complex formation and phosphorylation of β-catenin**.** Therefore, β-catenin can be translocated to the nucleus and bind to the TCF/LEF, which initiates the transcription.

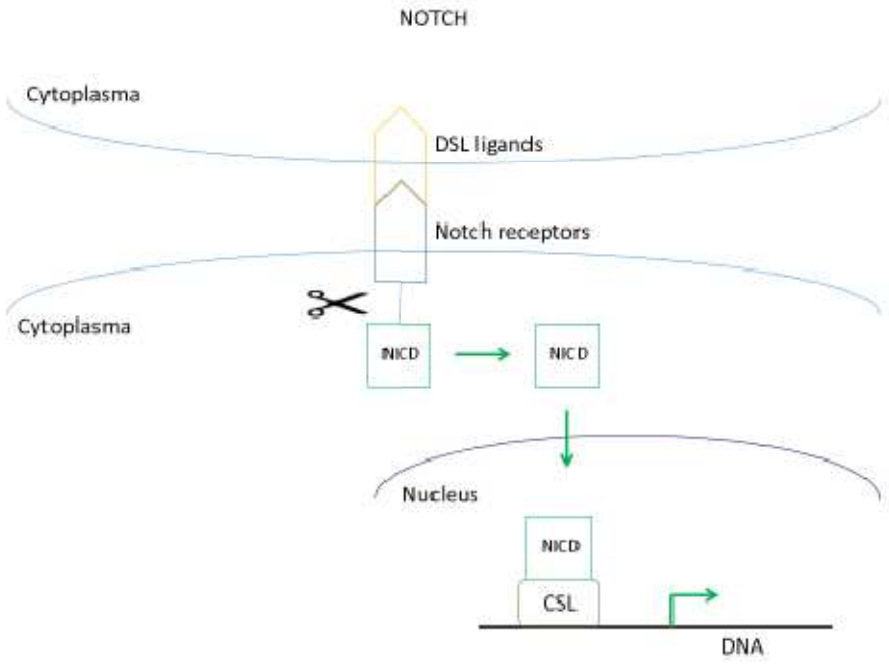

Figure 1.2 A Diagram of the Canonical Notch Signaling Pathway
Notch receptors and ligands are both expressed in the cell membranes. Upon the binding of Notch ligands, Notch receptors can be cleaved by metalloproteases and γ-secretases, which produces the active Notch intracellular domain, named as NICD. NICD can be translocated into nucleus and bind to CSL, which converts CSL from transcription repressor to transcription activator and subsequently initiates the gene transcription.

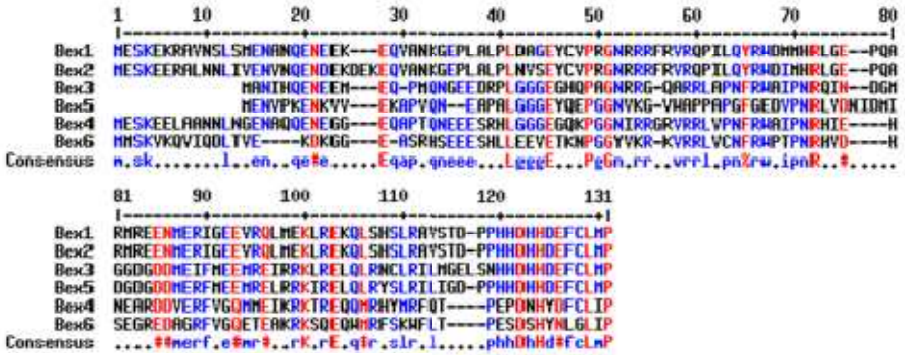

Figure 1.3 Alignment of the amino acid sequences of Bex proteins
Shown are the amino acid sequences comparison of human Bex1, Bex2, Bex3, Bex4, Bex5 and mouse Bex6. Red color represents high consensus, Blue color represents low consensus, and black represents neutral. Figures generated from the multialin website, http://multalin.toulouse.inra.fr/multalin/.

CHAPTER 2. NOTCH SIGNALING DEFICIENCY UNDERLIES AGE-DEPENDENT DEPLETION OF SATELLITE CELLS IN MUSCULAR DYSTROPHY

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2.1 Abstract

Duchenne Muscular Dystrophy (DMD) is a devastating disease characterized by muscle wasting, loss of mobility and early death. Satellite cells are muscle-resident stem cells responsible for the repair and regeneration of damaged muscles. One pathological feature of DMD is the progressive depletion of satellite cells, leading to the failure of muscle repair. Here I attempted to explore the molecular mechanisms underlying satellite cell ablation in the Dystrophin mutant MDX mouse, a well-established model for DMD. Initial muscle degeneration activates satellite cells, resulting in increased satellite cell number in young MDX mice. This is followed by rapid loss of satellite cells with age due to reduced self-renewal ability of MDX satellite cells. In addition, satellite cell composition is altered even in young MDX mice, with significant reductions in the abundance of non-committed Pax7+/Myf5- satellite cells. Using a Notch-reporter mouse, I found that the MDX satellite cells have reduced activation of Notch signaling, which has been shown to be necessary to maintain satellite cell

quiescence and self-renewal. Concomitantly, the expression of Notch1, Notch3, Jag1, Hey1 and HeyL are reduced in the MDX primary myoblast. Finally, I established a mouse model to constitutively activate Notch signaling in satellite cells, and show that Notch activation is sufficient to rescue the self-renewal deficiencies of MDX satellite cells. These results demonstrate that Notch signaling is essential for maintaining the satellite cell pool and its deficiency leads to depletion of satellite cells in DMD.

Keywords: Muscular Dystrophy, Notch Signaling, Stem cells

2.2 Introduction

Muscular dystrophies include a spectrum of inherited diseases leading to progressive muscle degeneration and dysfunction (Wallace and McNally, 2009). The most severe and common form of muscular dystrophy is Duchenne Muscular Dystrophy (DMD). DMD is a devastating recessive X-linked muscle degenerative disease caused by frame shift deletions, duplications, or point mutations in the *Dystrophin* (*DMD*) gene (Hoffman et al., 1987; Worton et al., 1984). Dystrophin is a cytoskeletal protein that interacts with a group of peripheral membrane and transmembrane proteins, such as dystroglycan and sarcoglycan, to form the dystrophin-associated protein complex (DAPC) (Matsumura et al., 1994). The DAPC provides a linkage between the cytoskeleton and extracellular matrix of muscle fibers, and maintains the integrity of sarcolemma (muscle membrane) during muscle contraction (Ervasti and Sonnemann, 2008). Absence of Dystrophin results in the disassociation of the DAPC. As a consequence, the sarcolemma becomes fragile to mechanical damages, and normal muscle activity would result in muscle degeneration, chronic inflammation and fibrosis (Petrof et al., 1993). These pathological stimulations alter the tissue environment and compromise muscle function to further deteriorate the dystrophic phenotype. DMD patients typically suffer from rapid progression of muscle degeneration, and are eventually paralyzed and die in their second to third decade of life.

Skeletal muscles have a remarkable capacity to regenerate. This capacity is mainly attributed to a stem cell population called satellite cells. Satellite cells

are muscle-specific adult stem cells responsible for muscle regeneration in response to injuries . Upon a muscle injury, satellite cells, located between the basal lamina (i. e. the muscle extracellular matrix) and sarcolemma, are activated from quiescence and proliferate as myogenic precursor cells, the proliferating myoblasts then undergo either self-renewal and return to quiescence, or differentiation to form functional muscles (Anderson, 2006; Cheung and Rando, 2013; Kuang and Rudnicki, 2008; Wagers and Conboy, 2005). In healthy humans, satellite cells can proliferate and repair muscle damage. However, unrelenting muscle degeneration in DMD puts satellite cells in a constant activation mode and eventually depletes the satellite cell pool, leading to the failure of muscle repair and accelerated disease progression (Blau et al., 1983; Blau et al., 1985; Heslop et al., 2000).

Currently, there is no effective treatment for DMD patients. To date, stem cell – based therapeutic strategies are under intense investigations. The stem cell therapies mainly include delivery of exogenous muscle stem cells to boost the regeneration of DMD muscles, and functional enhancement of endogenous muscle stem cells (Bentzinger et al., 2010; Quattrocelli et al., 2010; Shi and Garry, 2006). However, stem cell therapies are still in their infancy and to achieve the full potential of these regenerative approaches, it is necessary to better understand the cellular and molecular mechanisms governing satellite cell behavior and function. Previous studies have shown that Notch signaling pathway plays important roles in maintaining satellite cell quiescence as well as regulating proliferation and differentiation (Bjornson et al., 2012; Buas and

Kadesch, 2010; Conboy and Rando, 2002; Mourikis et al., 2012). Constitutive activation of Notch pathway promotes the self-renewal of satellite cells by up-regulating Pax7, a key regulator of satellite cell identity (Wen et al., 2012). Conversely, blockage of Notch signaling in satellite cells results in muscular dystrophy characteristics and impairs muscle regeneration (Lin et al., 2013). However, whether Notch signaling is deregulated in satellite cells of dystrophic muscles and whether it contributes to the progression of muscle degeneration have not been determined.

In this study, I aimed to address these questions using the MDX mouse model (Bulfield et al., 1984), which carries a mutation in the *Dmd* gene and thus has been widely used as an animal model for human DMD (Partridge, 2013). I discovered that MDX satellite cells exhibit defective self-renewal capacity associated with attenuated Notch signaling transduction. Importantly, constitutive activation of Notch signaling in the MDX satellite cells rescued their self-renewal defects. These data demonstrate that the attenuated Notch signaling in MDX leads to satellite cell dysfunction, and further suggest that Notch signaling preserves therapeutic potential to retain the self-renewal capacity in dystrophic muscles.

2.3 Materials and Methods

2.3.1 Animals

Myf5^{nLacZ} mice were provided by Shahragim Tajbakhsh (Christov et al., 2007). All other mice are available from Jackson Laboratories (ROSA26-N1ICD stock number 008159 and MDX stock number 001801, Pax7-CreER stock

number 012476, CpGFP stock number 005854). Mice were maintained in a clean mouse facility at Purdue University. All procedures involving animal maintenance and experimental use were performed according to the guidelines presented by Purdue University's Animal Care and Use Committee.

2.3.2 Muscle Injury and Regeneration

Muscle regeneration was induced by Cardiotoxin (CTX; Sigma-Aldrich, St. Louis, MO) injection. Mice were first anesthetized using a ketamine-xylazine cocktail, and then 50ul of 10 mM CTX was injected into the Tibialis Anterior (TA) muscle. Muscles were harvested at 7 days post injection.

2.3.3 Electroporation of DNA Plasmids into TA Muscles

About 10 µl of empty pEF-BOS Neo plasmid, Jag1 plasmid, or RAMIC domain (NICD) plasmid were injected at a concentration of 0.5 µg/µl (in 0.9% NaCl) into TA muscles along the whole muscle length. Two spatula electrodes were then placed on each side of the muscle belly, and eight pulses (20 ms 200 V/cm) at 1 second intervals were applied to the electrodes controlled by a BTX ECM 830 electroporator (Genetronics, San Diego, CA). The dosage was established in a preliminary study using the GFP plasmid, in which ~70% myofibers in the vicinity of the injection site and ~30% fibers in the whole muscle were GFP^+ at 1 week after electroporation. Using this protocol, Jag1 or RAMIC was electroporated into the left TA muscles of the Cp-GFP mice, and the empty pEF-BOS vector was electroporated into the contralateral (right) TA as control. One week after electroporation, the TA muscles were harvested for assessment of Cp-GFP and Pax7 expression by immunohistochemistry.

2.3.4 Isolation and Culture of Single Fibers and Primary Myoblast

Single myofibers were isolated from the Extensor Digitorum Longus (EDL) muscles after collagenase A (Sigma) digestion for 45 min to 1 hour. Suspended fibers were collected and cultured in horse serum-coated plates in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT), 2% chicken embryo extract (Accurate Chemical, Westbury, NY), and 1% penicillin-streptomycin for 72hs.

Primary myoblast was collected from limb skeletal muscle. These muscles were minced and digested with a cocktail of type I collagenase and Dispase B mixture. Debris was removed using filters from cells. Primary myoblast was cultured in 100mm collagen coated plates in the growth medium (F-10 Ham's medium supplemented with 20% FBS, 4 ng/ml basic fibroblast growth factor, and 1% penicillin-streptomycin) at 37℃, 5% CO_2.

2.3.5 Fluorescent Activated Cell Sorting

Cells were isolated from hindlimb muscles of 6- to 8-week-old mice. Erythrocytes were removed through the Red Blood Cell Lysing Buffer Hybri-Max (Sigma). Mononuclear cells were blocked with goat serum for 10 min and incubated with primary antibodies in DMEM with 2% FBS at 1–3 × 10^7 cell/ml for 15 min at 4℃. Cells were briefly washed and incubated with appropriate secondary antibodies (1: 1000) at 4℃ for 15 min. After staining, cells were washed, passed through 30 μm filters (Miltenyi Biotec) and suspended at a

concentration of 1 $\times 10^7$ cells/ml. Cells were separated on a MoFlo cytometer (DakoCytomation) equipped with three lasers. Sorting gates were strictly defined based on single antibody-stained control cells as well as the forward and SSC patterns of satellite cells based on preliminary tests.

2.3.6 Cryosection

Fresh TA muscles were embedded in optimal cutting temperature (OCT) compound (Sakura Finetek) and immediately frozen in dry ice-cooled isopentane. Muscle blocks were cut by 10um with a Leica CM 1850 cryostat instrument. The sections were placed on Superfrost Plus glass slides (Electron Microscopy Sciences).

2.3.7 Immunostaining and Image Capture

Muscle fibers and tissue sections were first fixed in 4% PFA and blocked in the blocking buffer containing PBS, 5% horse serum, 2% bovine serum albumin, 0.2% Triton X-100 and 0.1% sodium azide for 60mins. Then the fibers and sections were incubated with primary antibodies diluted in blocking buffer overnight at 4℃, then incubated with secondary antibodies and Hoechst diluted in PBS for 30 minutes at room temperature, and mounted with Dako fluorescent mounting media (Glostrup, Denmark). Fluorescent pictures were taken with a Coolsnap HQ CCD camera (Photometrics, USA) driven by IP Lab software (Scanalytics, USA) in a Leica DMI 6000B fluorescent microscope (Mannheim, Germany). As the analysis of the immunofluorescence was qualitative, identical

image handling and fluorescence scoring criteria were applied in all the experiments.

2.3.8 Quantitative Real-time Polymerase Chain Reaction (qPCR)

RNA was extracted and purified from wild type and MDX primary myoblast cell cultures using Trizol. Random hexamer primers were used for the reverse transcription from RNA to cDNA. qPCR was performed with a Light Cycler 480 machine (Roche). 18s was used as housekeeping gene for normalization. For qPCR result analysis, $2^{-\Delta\Delta ct}$ method was applied to calculate the fold change.

2.3.9 Statistical Analysis

The data are displayed with mean ± s.e.m. P-values were calculated by two-tailed Student's t-test. P-values <0.05 were considered to be statistically significant.

2.4 Results

2.4.1 Satellite Cell number and Activity Decline with Age in MDX Mice.

As satellite cells are necessary for postnatal muscle regeneration (Lepper et al., 2011; Murphy et al., 2011; Sambasivan et al., 2011), I aimed to examine satellite cell behavior in MDX mice whose muscles are under repetitive degeneration and regeneration. I first examined the abundance of satellite cells associated with freshly isolated myofibers from the Extensor Digitorum Longus (EDL) muscles of WT and MDX mice at different ages (**Figure 2.1A**). Interestingly, there were significantly more $Pax7^+$ satellite cells per myofiber in the MDX than WT mice at 2-, 6- and 12-month-old (**Figure 2.1B**). Whereas the

number of WT satellite cells continually decline with age at a slow rate, the MDX satellite cell number initially increases from 1-month-age to 6-month-age, followed by rapid decline afterwards (**Figure 2.1B**). As the severity of muscle pathology starts at approximately 2 months of age in mice (Bulfield et al., 1984), the initial increases in satellite cell number reflect the activation of satellite cells due to ongoing muscle injuries. The rapid decline of satellite cell number starting at 6 months of age suggests that the MDX satellite cells are unable to maintain a proper balance of proliferation, self-renewal and differentiation.

I further examined the proliferative activity of satellite cells in vivo and in vitro. In response to cardiotoxin (CTX)-induced muscle degeneration, satellite cells are activated and proliferate, then fuse to repair the injury. At Day 5 post CTX injection into TA muscles, the number of satellite cells per myofiber increased in both WT and MDX mice at 1-6 months of age (**Figure 2.1C**). However, the CTX-stimulated fold increases of satellite cells rapidly decreased with age in the MDX, but not in the WT mice (**Figure 2.1D**). These results indicate severe age-dependent deficiencies in the activation and/or proliferation of satellite cells in the MDX mice. I also cultured satellite cells while they are still attached on their host myofibers that were singly dissociated from 12 and 24-month-old mice (**Figure 2.1E**). After 72h in culture, the WT satellite cells proliferated and formed clusters of cells, but the MDX satellite cells in both 12 and 24-month-old mice failed to form cell clusters (**Figure 2.1F**). This observation confirms that satellite cell activity declines dramatically in aged MDX mice.

2.4.2 MDX Satellite Cells Have Reduced Self-renewal Capacity.

Self-renewal is a defining feature of all stem cells and necessary for maintaining the homeostasis of stem cells. I hypothesized that the age-dependent depletion of satellite cells in the MDX mice is due to a reduced self-renewal capacity. To test the hypothesis, I established the *Myf5*^{nLacZ}/*MDX* mice by breeding MDX mice with *Myf5*^{nLacZ} mice that marks Myf5-expressing cells by nuclear localized β-galactosidase (Christov et al., 2007). Previous studies have shown that Pax7^{+}Myf5^{-} satellite cells give rise to Pax7^{+}Myf5^{+} satellite cells during myogenic commitment, and the Pax7^{+}Myf5^{-} cells have higher self-renewal capacity (Kuang et al., 2007). I isolated EDL myofibers from WT (*Myf5*$^{+/+}$/*MDX*) and MDX (*Myf5*$^{nLacZ/+}$/*MDX*) mice that were injected with CTX to assure satellite cells in both WT and MDX mice were in the same activated state. Satellite cells were then labeled with antibodies to Pax7 and β-gal (**Figure 2.2A**). In the absence of CTX-induced injury, similar proportions of Pax7^{+}Myf5^{-} (β-gal^{-}) satellite cells were found between WT and MDX mice, at both 2 and 6 months of age (**Figure 2.2B**). After CTX-induced muscle injury, the abundance of Pax7^{+}Myf5^{-} satellite cells was reduced in the MDX mice at both 2- and 6-month-old (**Figure 2.2C**). Furthermore, the CTX-induced fold change of Pax7^{+}Myf5^{-} satellite cells was decreased in MDX compared to WT mice at both 2 and 6 months of age (**Figure 2.2D**). These in vivo data demonstrate that there is a self-renewal defect in MDX satellite cells.

I further examined satellite cell self-renewal using a well-established paradigm involving culture of dissociated myofibers (Halevy et al., 2004; Olguin

and Olwin, 2004; Zammit et al., 2004). After culture, the self-renewal, proliferation and differentiation progenies can be distinguished as $Pax7^{+}MyoD^{-}$, $Pax7^{+}MyoD^{+}$ and $Pax7^{-}MyoD^{+}$, respectively, based on their Pax7 and MyoD expression pattern (**Figure 2.2E**). Quantitative analysis indicates that the percentage of $Pax7^{+}MyoD^{-}$ (self-renewal) cells was drastically decreased in MDX myofiber cutlures (37% in WT vs 20% in MDX), while the percentage of $Pax7^{+}MyoD^{+}$ (proliferating) cells was increased in the MDX and the percentage of $Pax7^{-}MyoD^{+}$ (differentiating) cells was not different between WT and MDX (**Figure 2.2F**). Moreover, the expression of MyoG, a terminal differentiation marker of myogenesis, was examined after 72 hours myofiber culture (**Figure 2.2G**). Quantitative analysis revealed that the ratio of $MyoG^{+}/MyoD^{+}$ cells was elavated in MDX at both 2 and 6 months of age (**Figure 2.2H**), suggesting that MDX satellite cells have a higher tendency for terminal differentiation. Collectively, these cell culture data are consistent with the notion that the MDX satellite cells have reduced self-renewal capacity.

2.4.3 The Notch Signaling Pathway Is Perturbed in MDX Satellite Cells

To understand the molecular mechanism underlying the reduced self-renewal capacity in the MDX satellite cells, I investigated the Notch signaling pathway, which has been shown to mediate satellite cell self-renwal and quiescence (Vasyutina et al., 2007). I detected that the expression of genes related to Notch signaling pathway was reduced dramatically at both young (**Figure 2.3A**) and old (**Figure 2.3B**) MDX muscles. Strikingly, the reduction was much more robust in the old (12-month-old) MDX mice (**Figure 2.3B** compared

to **Figure 2.3A**). The reduced expression of Notch receptors, ligands and target genes in MDX muscles indicate an impairment of Notch signaling transduction.

To directly visualize the activation status of Notch signaling in satellite cells, I used the Cp-GFP reporter mouse (Mizutani et al., 2007). In this transgenic mouse, GFP expression is driven by 4 tandem repeats of DNA sequence recognized by Rbpjk, the nuclear mediator of Notch signaling. When Notch is not activated, Rbpjk binds to transcriptional repressors which suppress GFP expression. Upon activation (ligand binding), Notch intracellular domain (NICD) would detach and translocate to the nucleus, where it replaces the transcriptional repressors on Rbpjk and activates GFP expression. To establish the utility of this model in satellite cells, single myofibers were isolated from the Cp-GFP mice and GFP and Pax7 expression was examined (**Figure 2.3C-a**). A fraction (~17%) of $Pax7^+$ satellite cells was GFP^+ (**Figure 2.3C-b**). Next, fluorescent activated cell sorting (FACS) was used to isolate GFP^+ and GFP^- satellite cells using α7-Integrin (Int-α7) as a positive selection marker for satellite cells (**Figure 2.3C-c**). Analysis of FACS-purified satellite cells indicates that 8% freshly sorting satellite cells ($Lin^-Int\text{-}\alpha7^+$) were also GFP^+ (**Figure 2.3C-d**). Importantly, the FACS-purified GFP^+ satellite cells expressed higher levels of *Hes1* (**Figure 2.3C-e**), a canonical target of Notch signaling pathway. To further examine if the Cp-GFP reporter responds to Notch activation in vivo, the DNA plasmids encoding a Notch ligand (Jag1) or activated Notch (NICD, also called RAMIC domain) were electroporated in the TA muscles, and Pax7 and GFP expressions were analyzed 7 days later (**Figure 2.3C-f to Figure 2.3C-h**). Overall, Jag1

overexpression increased the percentage of $Pax7^+/GFP^+$ satellite cells by ~30% (n=1,191 cells analyzed) and NICD overexpression increased the double positive satellite cells by ~50% (n= 825 cells analyzed). Thus, the Cp-GFP reporter mouse faithfully reports Notch signaling activation in satellite cells. The single fiber and FACS analyses further demonstrate that Notch signaling is activated in a small population of quiescent satellite cells.

I next established the *Cp-GFP*/*MDX* mouse model through crossing the two lines of mice and examined Cp-GFP expression. EDL myofibers were isolated from WT control (Cp-GFP/WT) and MDX (*Cp-GFP*/*MDX*) mice 5 days after CTX-induced regeneration, and labeled with Pax7 and GFP antibodies (**Figure 2.3D**). Notably, the percentage of GFP^+ satellite cells (% GFP^+Pax7^+/total $Pax7^+$) in MDX was only about half of that in WT mice after CTX injury(Figure 2. 3L; 37% in WT vs 20% in MDX). By contrast, there was no significant difference in the percentage of GFP^+ satellite cells between WT and MDX mice under resting conditions (**Figure 2.3E**). This result suggests that MDX satellite cells have reduced activation of Notch signaling during muscle regeneration. Consistent with this notion, mRNA levels of Notch receptors (*Notch1* and *Notch3*) and Notch ligand (*Jagged1*) as well as Notch target (*HeyL*) were reduced by ~50% in primary myoblasts derived from MDX mice compared to those from WT mice (**Figure 2.3F**). Taken together, the self-renewal defects of MDX satellite cells is associated with reduced Notch signaling transduction.

2.4.4 Constitutive Activation of Notch Rescues the Self-renewal Defects of Satellite Cells but Fails to Improve Muscle Pathology in MDX Mice

To directly address whether reduced Notch signaling in MDX satellite cells is responsible for their self-renewal defects, I carried out gain-of-function studies using Cre/LoxP mediated conditional gene expression tools. I first cultured EDL myofibers from *Rosa26*^{NICD}/*MDX* mice, and used Adenovirus-Cre to activate NICD expression during the culture. After 72 h of culture, myofibers were stained with Pax7 and MyoD (**Figure 2.4A**). Quantitative analysis indicates that the percentage of Pax7^{+}MyoD^{-} (self-renewal) satellite cells was drastically increased (from 12% to 32%) after Cre induced Notch activation, compared to the control Adenovirus-GFP treatment (**Figure 2.4B**). Meanwhile the ratio of proliferating (Pax7^{+}MyoD^{+}) cells was significantly decreased by Notch activation (**Figure 2.4B**). These results demonstrate that Notch activation improves self-renewal and inhibits the proliferation of satellite cells in MDX mice.

I next attempted to activate Notch signaling in MDX satellite cells in vivo. To do this, I established the *Pax7*^{CreER}/*Rosa26*^{NICD}/*MDX* triple transgenic mouse in which Notch signaling is specifically activated in MDX satellite cells upon Tamoxifen induction. After 5 doses of Tamoxifen injection, NICD (revealed by nuclear GFP expression as the *Rosa26*^{NICD} mice also expression nuclear GFP upon Cre treatment) was specifically activated in the Pax7^{+} satellite cells (**Figure 2.4C**). Analysis of TA muscle cross sections (**Figure 2.4D**) indicates that the number of Pax7^{+} satellite cells was increased significantly after Notch activation (**Figure 2.4E**). More strikingly, the percentage of Pax7^{+}MyoD^{-} (self-renewal)

satellite cells was also significantly increased (from 40% to 60%) after NICD overexpression (**Figure 2.4F**). These results provide compelling evidence that constitutive activation of Notch rescues the self-renewal defect of MDX satellite cells.

To examine how satellite cell-specific stimulation of Notch signaling affects muscle pathology in MDX mice, I conducted histological analysis of TA muscles sections with and without CTX injury. Surprisingly, Notch activation in satellite cells failed to ameliorate muscle pathology and improve CTX-induced muscle regeneration in the MDX mice (**Figure 2.4G**). This is manifested by the reduced number of myofibers with an area of 500μm^2 or greater (**Figure 2.4H**). This observation is consistent with our recent report that constitutive Notch activation in satellite cells blocked muscle regeneration in WT mice (Wen et al., 2012), due to the well-known function of Notch signaling in inhibiting myogenic differentiation (Kopan et al., 1994). These data suggest that although constitutive Notch activation promotes self-renewal of satellite cells and increases satellite cell number in MDX mice, Notch signaling must be temporally suppressed during myogenic differentiation to allow proper progression of myogenesis.

2.5 Discussions

In this work, I identify that the satellite cells in MDX mice undergoes drastic change not only in the cell number but also in the cell activity. Specifically, the satellite cells in dystrophic muscles were defective in self-renewal capacity. Also, I verify that the Notch signaling pathway was perturbed in MDX satellite cells

using Notch reporter mice. Furthermore, I show that constitutive activation of Notch signaling rescued the self-renewal deficiency of MDX satellite cells *in vitro* and *in vivo.* However, constitutive activation of Notch failed to improve muscle regeneration in MDX mice. Nevertheless, these results suggest that aberrant Notch signaling is responsible for the defective self-renewal capacity and enhancement of Notch signaling leads to improved self-renewal of satellite cells in muscular dystrophy.

Muscular dystrophy has been characterized as an inherited disease featuring susceptibility to muscle damage and progressive muscle wasting. Given the indispensable role of satellite cells in muscle regeneration (Sambasivan et al., 2011; von Maltzahn et al., 2013) , the behavior of satellite cells, especially their replicative and differentiation capacity, determines the progression of muscle dystrophies. Primary myoblasts isolated from DMD patients have a significant low yield (Blau, 1983) and reduced proliferative capacity (Lamperth et al., 1990). Correspondingly, primary myoblasts from MDX mice exhibits accelerated differentiation kinetics (Yablonka-Reuveni and Anderson, 2006), supporting the notion that the proliferative capacity was compromised in the satellite cells in dystrophic muscles. Our results that the satellite cell number and proliferative response to CTX-stimulation declined with age in MDX mice provide direct in vivo evidence for age-dependent deficiencies in satellite cell activity.

Our findings that satellite cells in dystrophic muscles are defective in self-renewal capability are consistent with the previous study that muscle dystrophy results from an autonomous failure of satellite cells to maintain repetitive

degeneration and regeneration cycles (Sacco et al., 2010). It is worth mentioning that murine somatic cells have longer telomeres compared to human cells, which significantly decreases the replicative senescence and increase the regenerative capacity of MDX satellite cells (compared to DMD satellite cells). To some extent, this explains why the MDX mice have slower pathological progression relative to human DMD. However, our results indicated that the satellite cells in MDX mice still exhibit deficiencies in self-renewal capability. Specifically, old MDX satellite cells cannot replenish themselves at all in 24-month-old, while the WT non-dystrophic satellite cells can still self-renewal (but at a significantly reduced rate compared to satellite cells from young animals). This finding prompted us to investigate the molecular mechanism behind the defective self-renewal capacity of MDX satellite cells. The mechanism governing the self-renewal of satellite cells is currently a topic of intense investigation. Recent studies have revealed a number factors that induce the quiescence/self-renewal of satellite cells. These include Sprouty1 (Shea et al., 2010), Angiopoietin 1 (Ang1) / Tie2 pathway (Abou-Khalil et al., 2009), Par-complex-dependent p38α/β MAPK pathway (Troy et al., 2012), microRNA-489 (Cheung et al., 2012), and nitric oxide (Buono et al., 2012). However, what signaling mechanism(s) regulate the self-renewal deficiency in MDX satellite cells has not been determined.

The Notch signaling pathway plays complicated and critical roles in embryonic muscle development and postnatal myogenesis. Previous studies have demonstrated an interaction between Dystrophin and Notch pathway. In *Drosophila*, the membrane Dystrophin/Dystroglycan complex can interplay with

the Notch ligand Delta (Kucherenko et al., 2008), which implies that the Notch signaling pathway may be perturbed in the absence of Dystrophin in MDX muscles. This notion was supported by the microarray analysis of dystrophic muscles. A number of key genes involved in the Notch signaling pathway have been detected with altered levels in dystrophic muscles. Specifically, the Notch antagonist *Numb* is up-regulated while the Notch target genes *Hes1* and *Hey1* are down-regulated drastically (Turk et al., 2005). Most recently, Church et al discovered a reduction of Notch1 and Hes1 mRNA level in the TA muscles of MDX mice and DMD patients (Church et al., 2014). However, direct in vivo evidence demonstrating an aberrant Notch signaling occurs in satellite cells in dystrophic muscles has been lacking. Using Cp-GFP as a reporter of the Notch signaling pathway activation, I now provide in vivo evidence that the Notch signaling pathway is perturbed in satellite cells of MDX muscles. This is the first direct *in viv*o evidence that the Notch signaling pathway was inhibited in satellite cells of dystrophic muscles. Our finding is consistent with the previous studies that Notch blockage in satellite cells can cause muscle dystrophic phenotype as well as deteriorated muscle regeneration (Lin et al., 2013). In addition, Church et al also show that Notch inhibition impeded the functional recovery of regenerated MDX muscles (Church et al., 2014). To rescue this self-renewal defect in dystrophic muscles, I established a mouse model to constitutively activate Notch signaling in satellite cells. As expected, Notch activation successfully ameliorated the self-renewal capacity, which corroborates that the impaired Notch signaling pathway contributes to the defective self-renewal capacity in the satellite cells of

dystrophic muscles. Therefore, Notch activation may provide a potential route to prevent premature depletion of satellite cells in DMD patients. Also, with the respective of the long-term treatment of muscle dystrophy, our studies suggest that the Notch inhibitor therapies may have potential side effects by accelerating satellite cells exhaustion. However, continuous Notch activation fails to improve regeneration of MDX muscles. I interpret this observation as the lack of myogenic differentiation due to the inhibition of *MyoD* and *myogenin* by Notch signaling. Taken together, a dynamic regulation of Notch signaling is necessary to balance self-renewal and differentiation of satellite cells in order to ameliorate the long-term regenerative defects of dystrophic muscles.

2.6 Acknowledgements

The authors would like to thank Shahragim Tajbakhsh (Pasteur Institute) for providing the *Myf5*^{nLacZ} mice; Signe Hobaugh and Jun Wu for maintaining mouse colonies and lab duties; and other members of the Kuang lab for technical assistance and discussion.

2.7 Competing Interests Statement

The authors declare no conflict of interests.

2.8 Author Contributions

S. K. and C. J. conceived and designed the experiments. C. J., Y. W., K. K. and S. K. performed the experiments. K. H. assisted muscle electroporation. M.

A. R. contributed reagents/materials and analysed data. C. J. and S. K. wrote the paper.

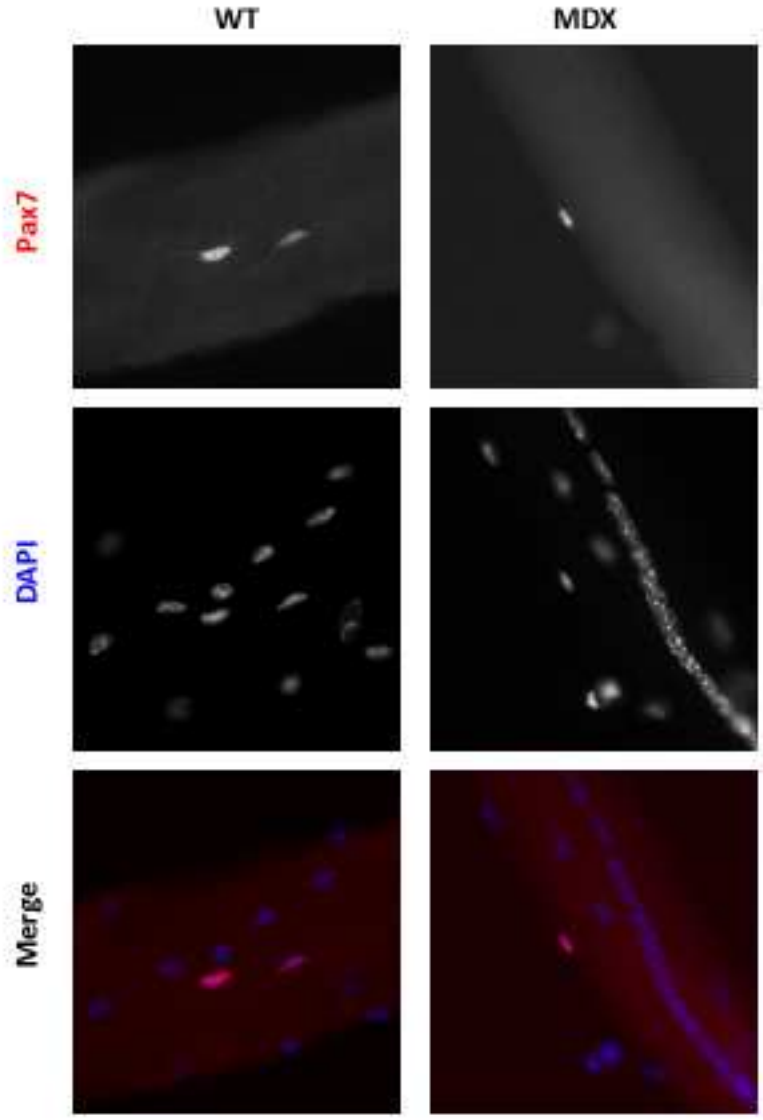

Figure 2.1 A Satellite Cells on Myofibers from WT and MDX Mice.
Single EDL muscle fibers were isolated from WT and MDX mice. Satellite cells attached on the fibers were labeled with Pax7 (red). Nuclei were counterstained with DAPI (blue). Central nuclei indicate MDX muscle regeneration.

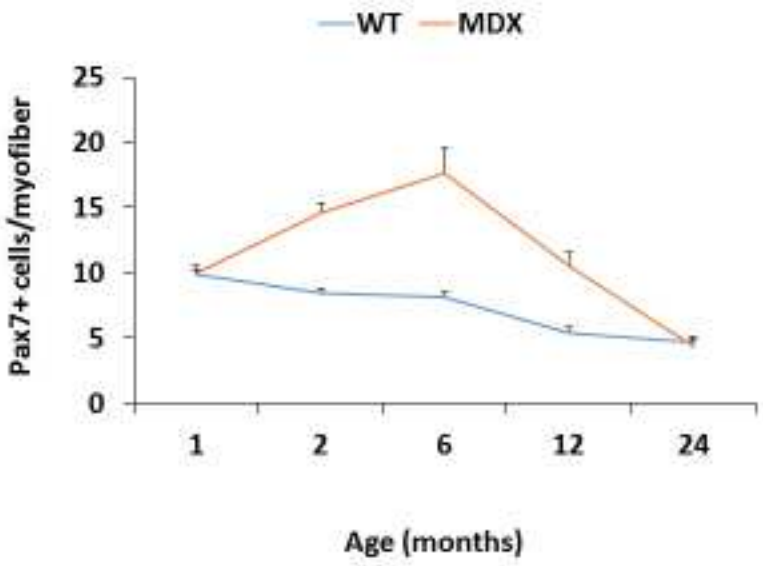

Figure 2.1 B Satellite Cell Number Declines with Age Rapidly in MDX mice. Satellite cell numbers in single EDL muscle fibers were quantified from WT and MDX mice at indicated ages. At each time point, n=3 mice with more than 20 fibers from each mouse were analyzed. Error bars represent s.e.m. * $P<0.05$.

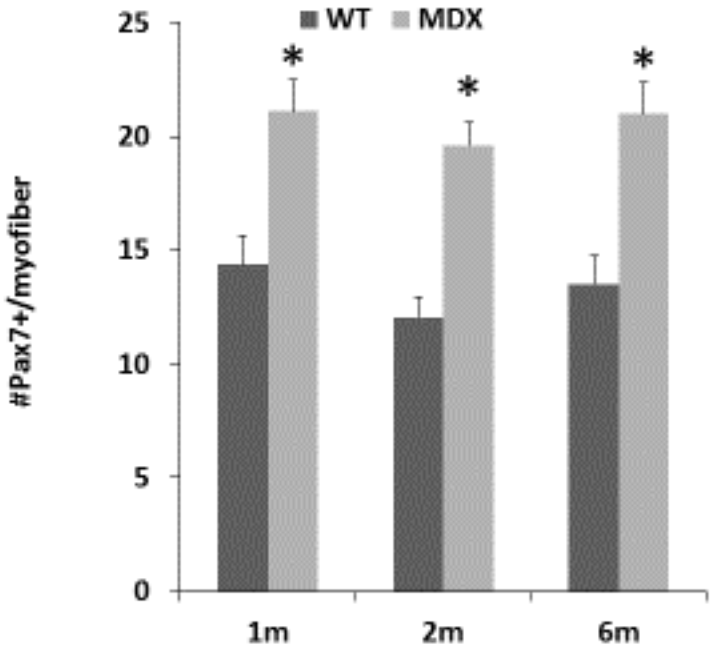

Figure 2.1 C Satellite Cell Number Increases More in MDX Mice after Injury. Satellite cell numbers in single EDL muscle fibers were quantified from WT and MDX mice at indicated ages after CTX treatment. At each ages, n=3 mice with more than 20 fibers from each mouse were analyzed. Error bars represent s.e.m.* $P<0.05$.

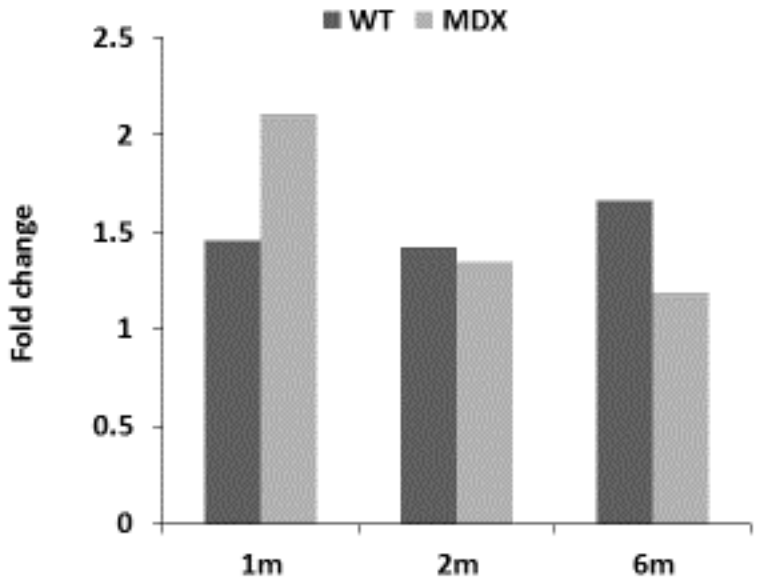

Figure 2.1 D CTX-stimulated Relative Fold Change of Satellite Cell Number Decreases with Age in MDX Mice.
Figure 2.1B showed the number of satellite cells without CTX injury while Figure 2.1C showed those numbers after CTX injury in WT and MDX mice at different age points (1m, 2m, and 6m). The fold change calculated in Figure 2.1D can be indicative of the responsiveness of satellite cells to the acute muscle injury.

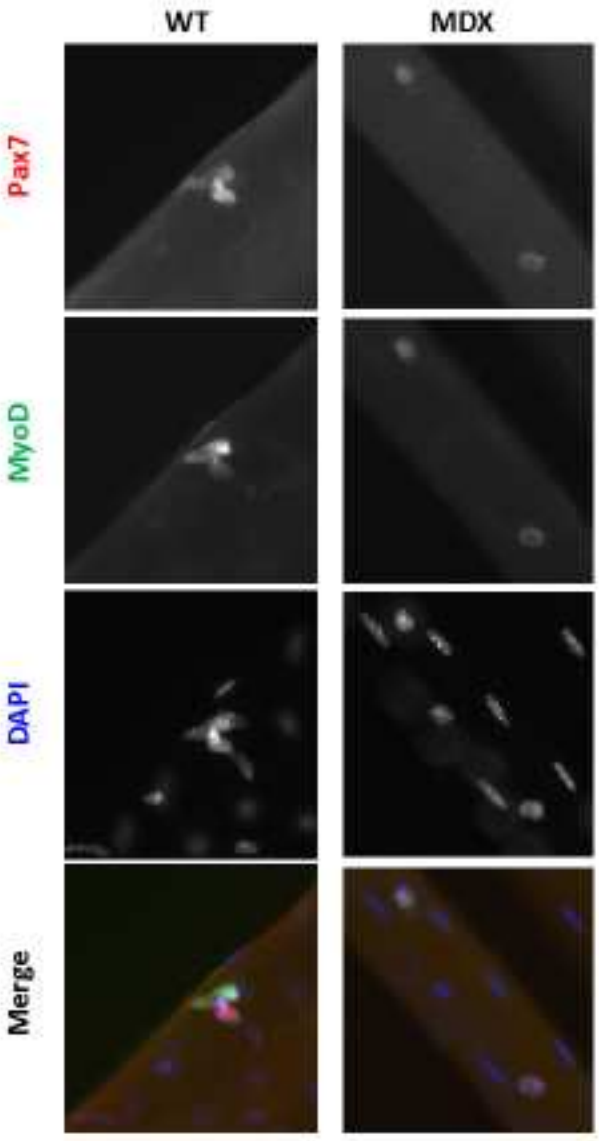

Figure 2.1 E Satellite Cells Failed to Form Clusters in Culture of Single Fibers from Aged MDX Mice.
Single fibers from 24 months old WT and MDX mice were cultured for 72hs. Satellite cell clusters were formed in the single fiber culture. Satellite cell were labeled with Pax7 (red) and MyoD (green). Nuclei were counterstained with DAPI (blue).

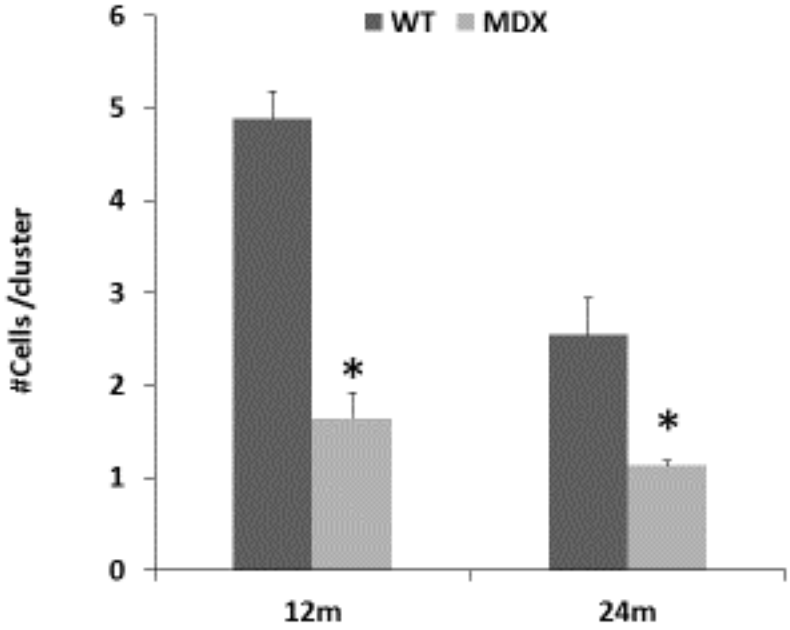

Figure 2.1 F The Number of Satellite Cells in One Cluster Was Reduced in Culture of Single Fibers from Aged MDX Mice.
Single fibers from 12 and 24 months old WT and MDX mice were cultured for 72hs. Satellite cell numbers in one cluster were quantified. n=3 independent experiment with more than 20 clusters analyzed in each experiment. Error bars represent s.e.m. *P<0.05.

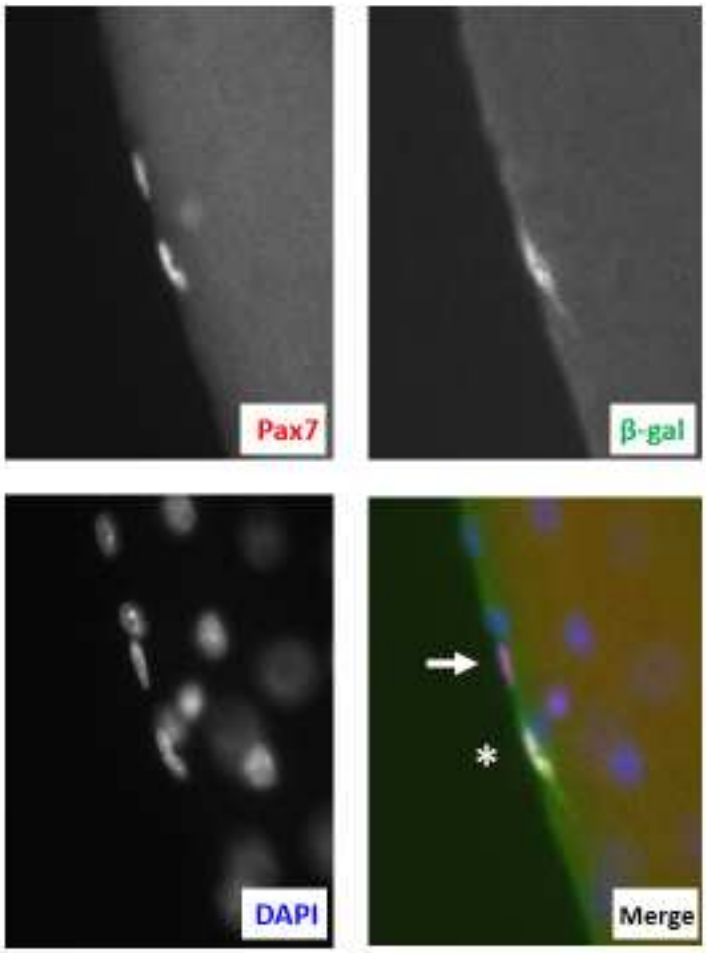

Figure 2.2 A A Subset of Satellite Cells Are Self-renewing.
Single EDL muscle fibers were isolated from WT and MDX mice. Satellite cells attached on the fibers were labeled with Pax7 (red) and β-gal (green). Pax7+β-gal+ and Pax7+β-gal- Satellite Cells were indicated by asterisk and arrow respectively. Nuclei were counterstained with DAPI (blue).

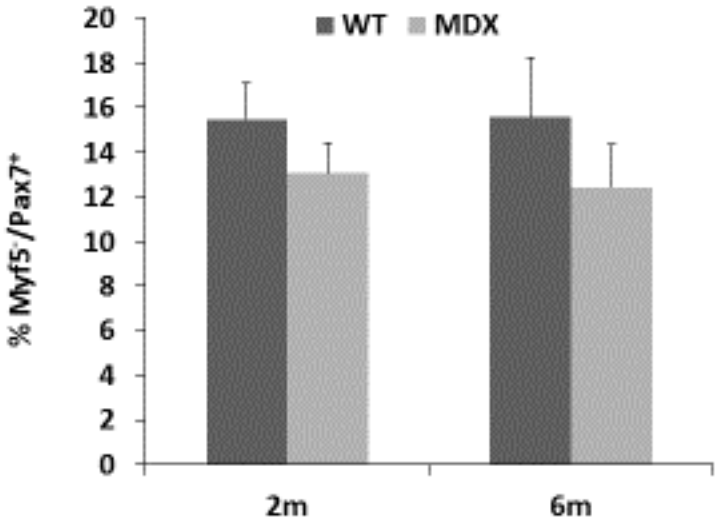

Figure 2.2 B The Abundance of Self-renewal Satellite Cells Is Normal in Non-injured MDX Muscles.
Single EDL muscle fibers were isolated from WT and MDX mice without CTX injury. Based on Pax7 and β-gal staining, percentages of self-renewal satellite cells (Pax7+Myf5- (β-gal-)) were quantified at the indicated ages. n=3 mice with more than 20 fibers analyzed in each mouse. Error bars represent s.e.m. *P<0.05.

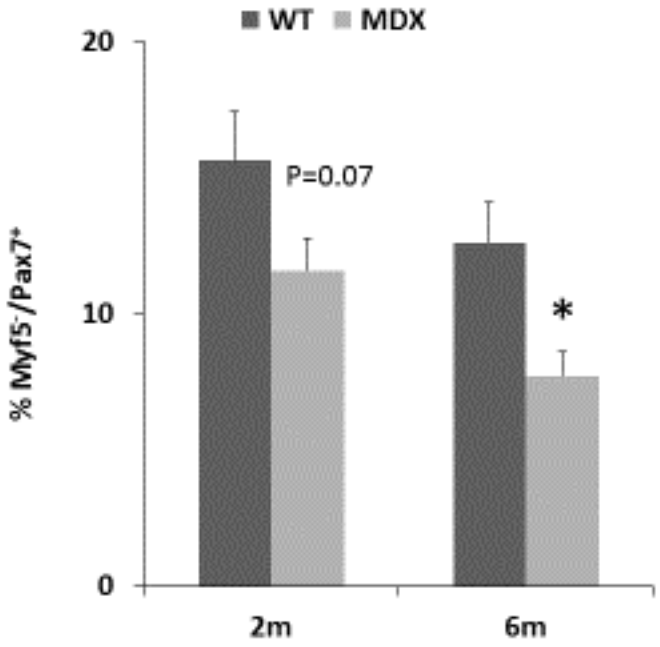

Figure 2.2 C The Abundance of Self-renewal Satellite Cells Decreases in MDX Muscles after Injury.
Single EDL muscle fibers were isolated from WT and MDX mice after CTX injury. Based on Pax7 and β-gal staining, percentages of self-renewal satellite cells (Pax7+Myf5- (β-gal-)) were quantified at the indicated ages. n=3 mice with more than 20 fibers analyzed in each mouse. Error bar represents s.e.m. *P<0.05.

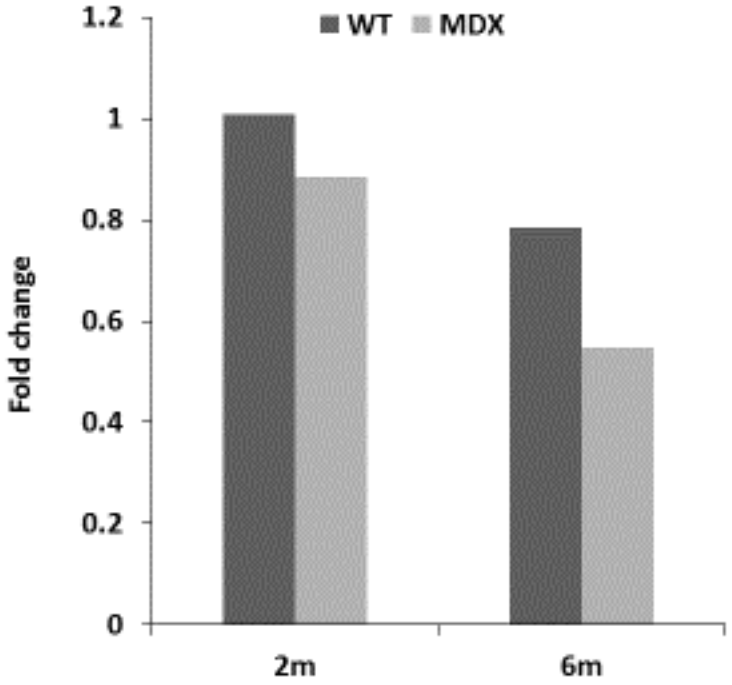

Figure 2.2 D CTX-stimulated Relative Fold Change of Self-renewal Satellite Cell Abundance Decreases in MDX Mice.
The CTX-stimulated relative fold change of self-renewal satellite cell abundance was calculated from data in Figure 2.2B and Figure 2.2C. The fold changes indicate the responsiveness of the self-renewal capability of satellite cells to the acute muscle injury.

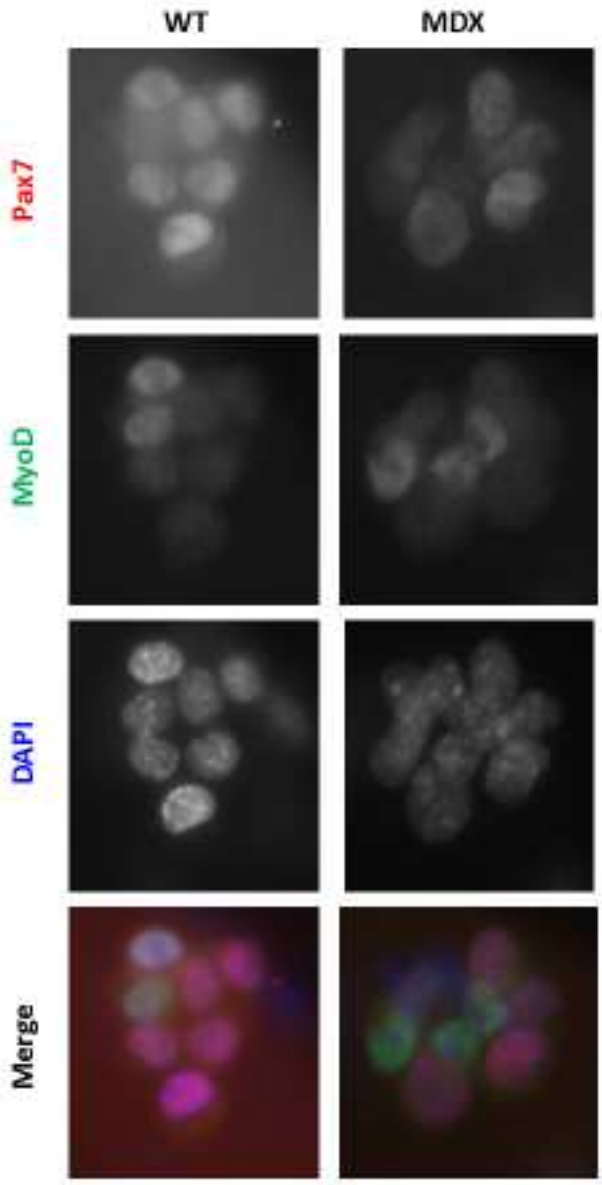

Figure 2.2 E Different Subpopulations in Satellite Cell Clusters Are Going through Self-renewal, Proliferation and Differentiation.
Single fibers from WT and MDX mice were cultured for 72hs. Satellite cell clusters were formed in the single fiber culture. Satellite cell were labeled with Pax7 (red) and MyoD (green). Quiescent, proliferating and differentiating cells were labeled by Pax7+MyoD-, Pax7+MyoD+, and Pax7-MyoD staining, respectively. Nuclei were counterstained with DAPI (blue).

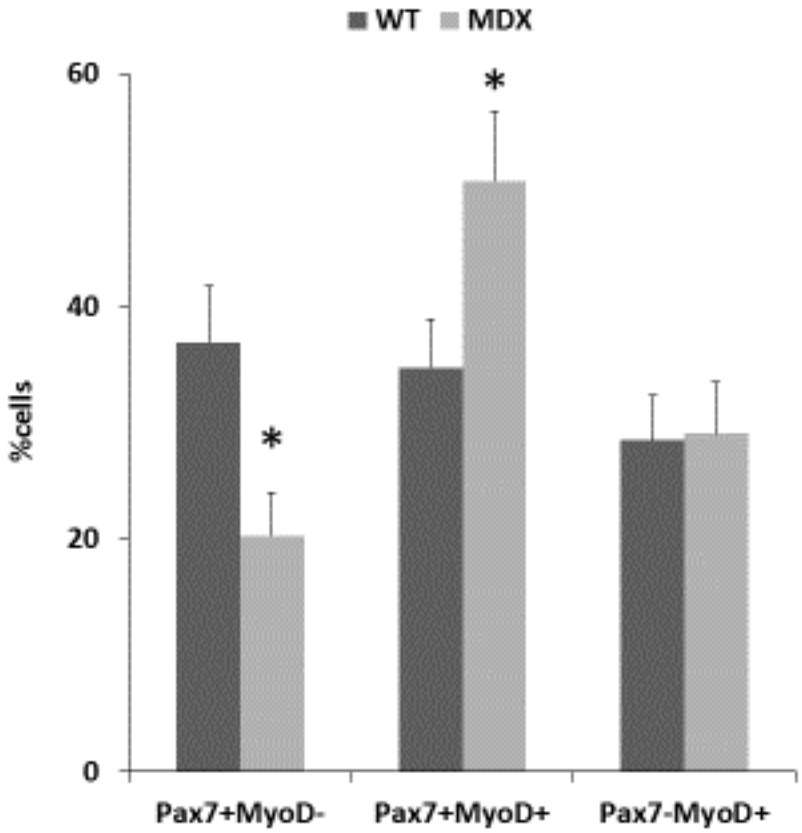

Figure 2.2 F Self-renewal Capacity Is Reduced in MDX Satellite Cells. Single fibers from WT and MDX mice were cultured for 72hs. Percentages of subpopulations of cells in one cluster were quantified based on Pax7 and MyoD staining. n=3 independent experiment with more than 20 clusters analyzed in each experiment. Error bars represent s.e.m. *P<0.05.

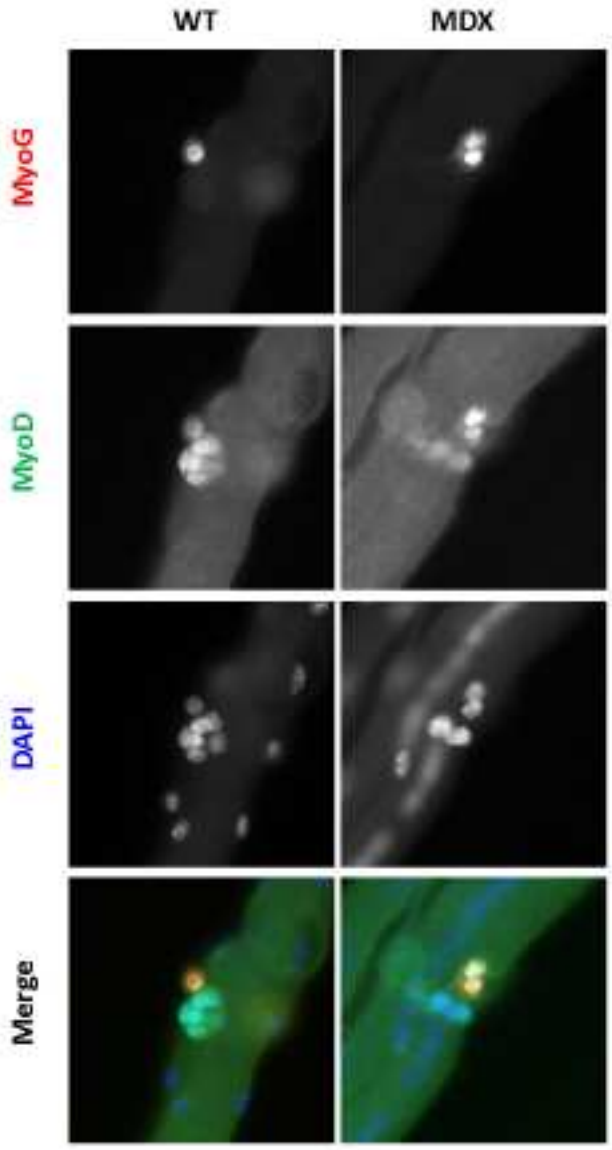

Figure 2.2 G A Subset of Satellite Cells in One Cluster Tends to Differentiate.
Single fibers from WT and MDX mice were cultured for 72hs. Satellite cell clusters were formed in the single fiber culture. Satellite cell were labeled with myogenin (red) and MyoD (green). Nuclei were counterstained with DAPI (blue).

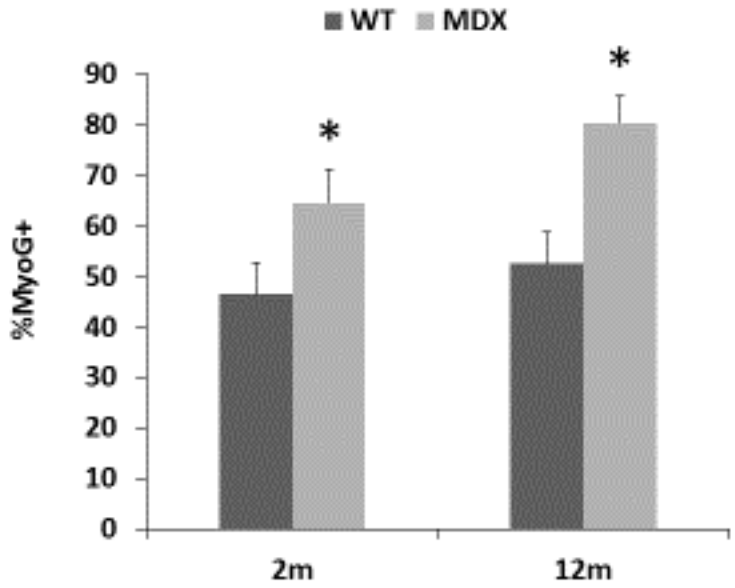

Figure 2.2 H MDX Satellite Cells Are More Prone to Terminal Differentiation. Single fibers from WT and MDX mice were cultured for 72hs. Percentages of myogenin+ cells per cluster were quantified based on myogenin and MyoD staining. n=3 independent experiment with more than 20 clusters analyzed in each experiment. Error bars represent s.e.m. *P<0.05.

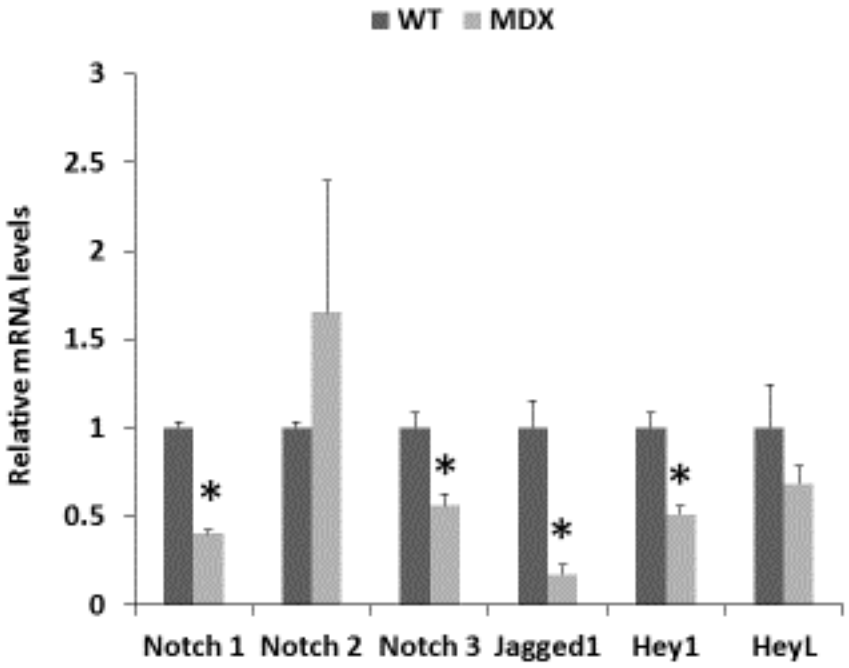

Figure 2.3 A The Notch Signaling Pathway Is Decreased in Young MDX Muscles.
RNA were extracted from TA muscles collected from WT and MDX mice at 2 months age. n=6 mice for each group. Error bars represent s.e.m. *P<0.05.

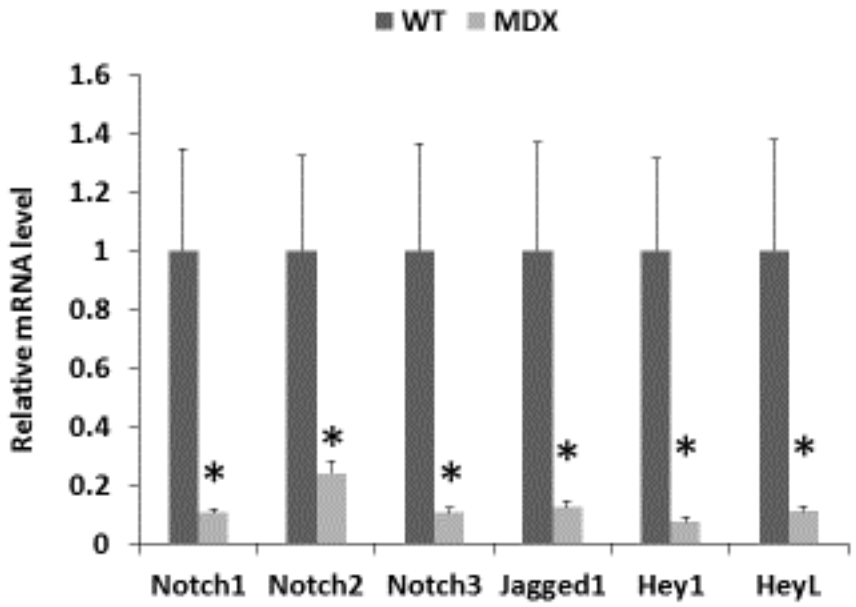

Figure 2.3 B The Notch Signaling Pathway Is Decreased in Aged MDX Muscles
RNA were extracted from TA muscles collected from WT and MDX mice at 12 months age. n=6 mice for each group. Error bars represent s.e.m. *P<0.05.

Figure 2.3 C The Cp-GFP Transgenic Mouse Is a Faithful Reporter of Notch Signaling in Satellite Cells.
(a) GFP signal in a putative satellite cell on a freshly isolated live EDL myofiber. (b) Double labeling with Pax7 (Red, satellite cell marker) and GFP (Green) indicates that 83% satellite cells (Pax7+) are GFP- (b1), but 17% are GFP+ (b2, n=102 cells counted from 3 mice). (c) Isolation of GFP+ and GFP- satellite cells from the Cp-GFP mouse by fluorescent activated cell sorting using a7-integrin as a positive selection marker. (d) Analysis of sorted a7-integrin+ cells indicates that 8% of cells are GFP+. (e) Realtime PCR analysis indicate that sorted GFP+ satellite cells expressed higher levels of Hes1, a canonical target of Notch signaling. (f-h) Activation of Notch signaling turns on Cp-GFP in satellite cells in vivo. Shown are images of Pax7 and GFP staining in control (f), Jag1 overexpressing (g), and RAMIC (NICD) overexpressing (h) TA muscle sections 7 days after electroporation of plasmids encoding empty vector, Jag1 cDNA and NICD cDNA, respectively. Arrows point to Pax7+/GFP+ satellite cells. Figure 2.3 C was conducted by Dr. Yefei, Wen, Dr. Kazuki Kuroda, and Dr. Shihuan Kuang.

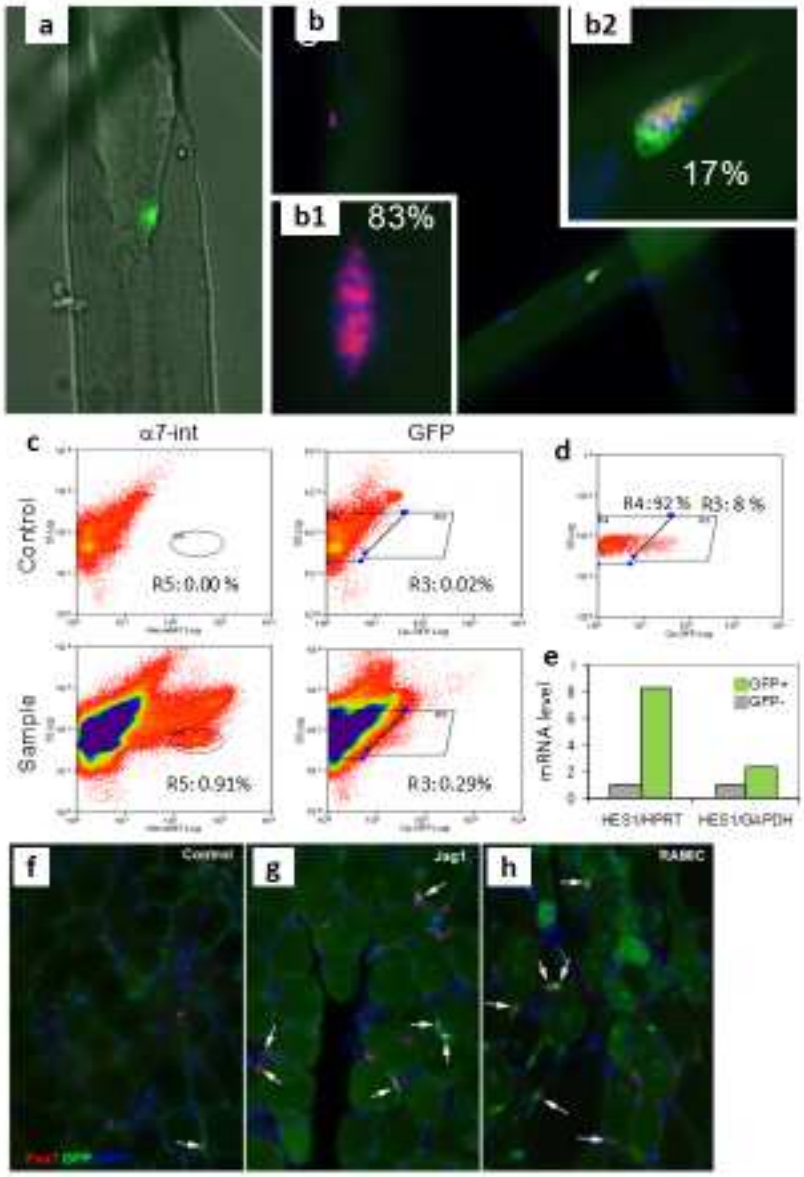

Figure 2.3 C The Cp-GFP Transgenic Mouse Is a Faithful Reporter of Notch Signaling in Satellite Cells.

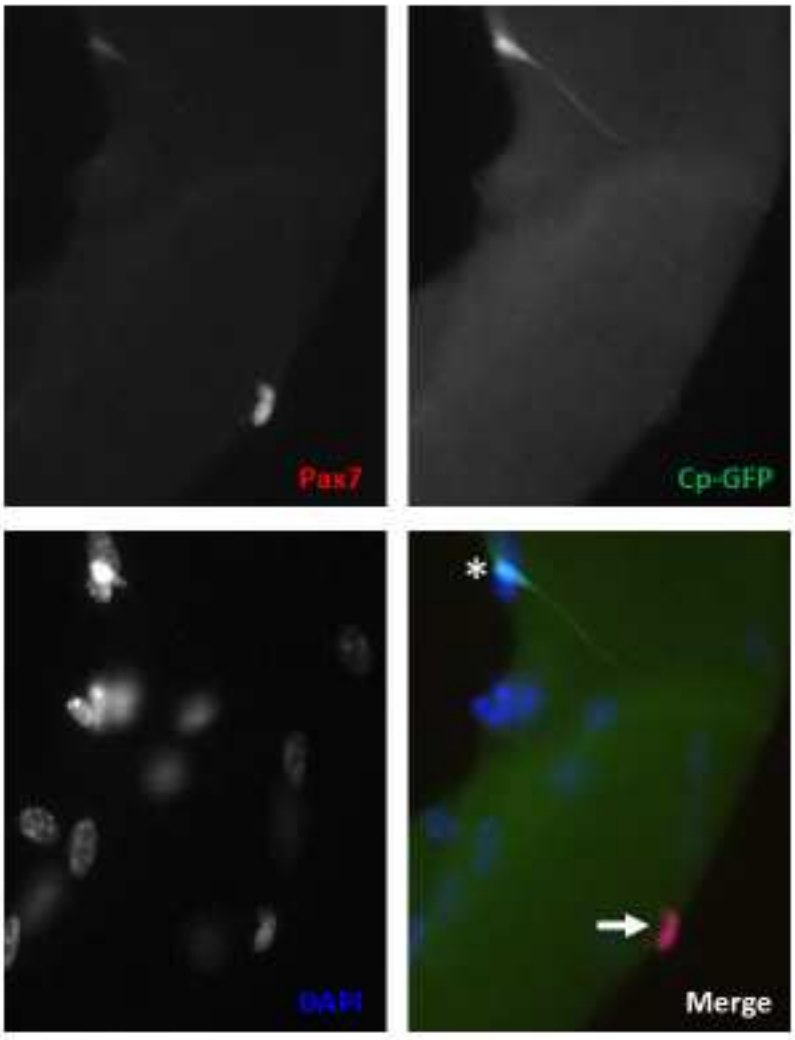

Figure 2.3 D The Notch Signaling Pathway Is Active in a Subset of Satellite Cells.
Single EDL muscle fibers were isolated from Cp-GFP/MDX mice. Satellite cells attached on the fibers were labeled with Pax7 (red) and GFP (green). The asterisk and arrow represent Pax7+GFP+ and Pax7+GFP- satellite cells, respectively. Nuclei were counterstained with DAPI (blue).

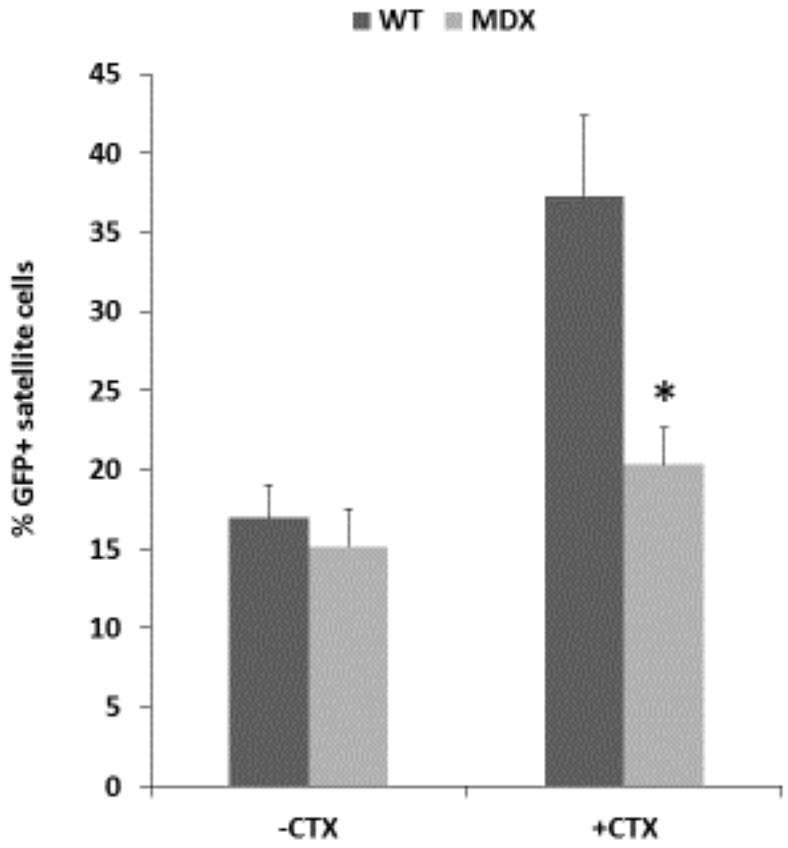

Figure 2.3 E The Notch Activity Is Decreased in MDX Satellite Cells after Injury.
Single EDL muscle fibers were isolated from WT and MDX mice without and with CTX injury. Based on Pax7 and GFP staining, percentages of Notch active satellite cells were quantified. n=3 mice with more than 20 fibers analyzed in each mouse. Error bars represent s.e.m. *P<0.05.

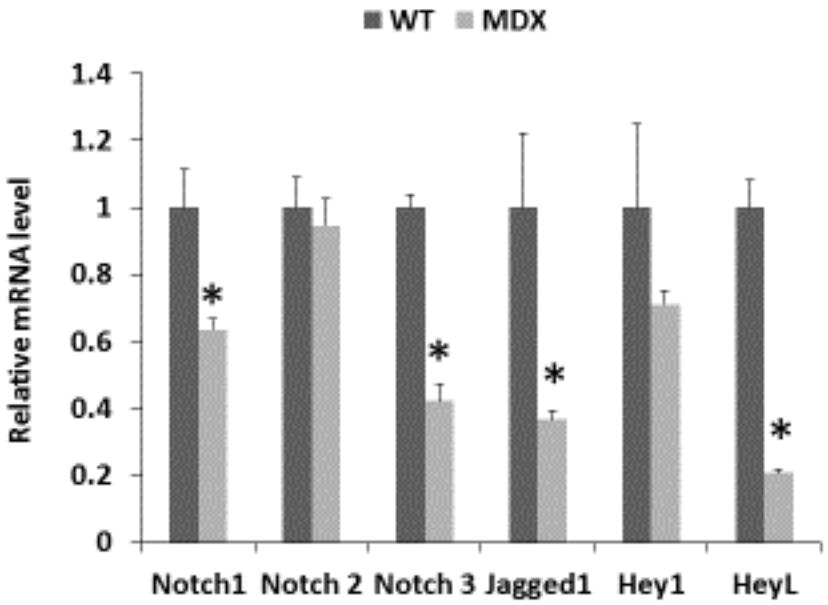

Figure 2.3 F The Notch Signaling Pathway Is Impaired in MDX Myoblasts. WT and MDX primary myoblast were cultured and collected for qPCR analysis including Notch receptors (Notch 1, 2, and 3) and ligand (Jagged1) as well as Notch target genes (Hey1 and HeyL). 3 independent experiments were performed. Error bars represent s.e.m. *P<0.05.

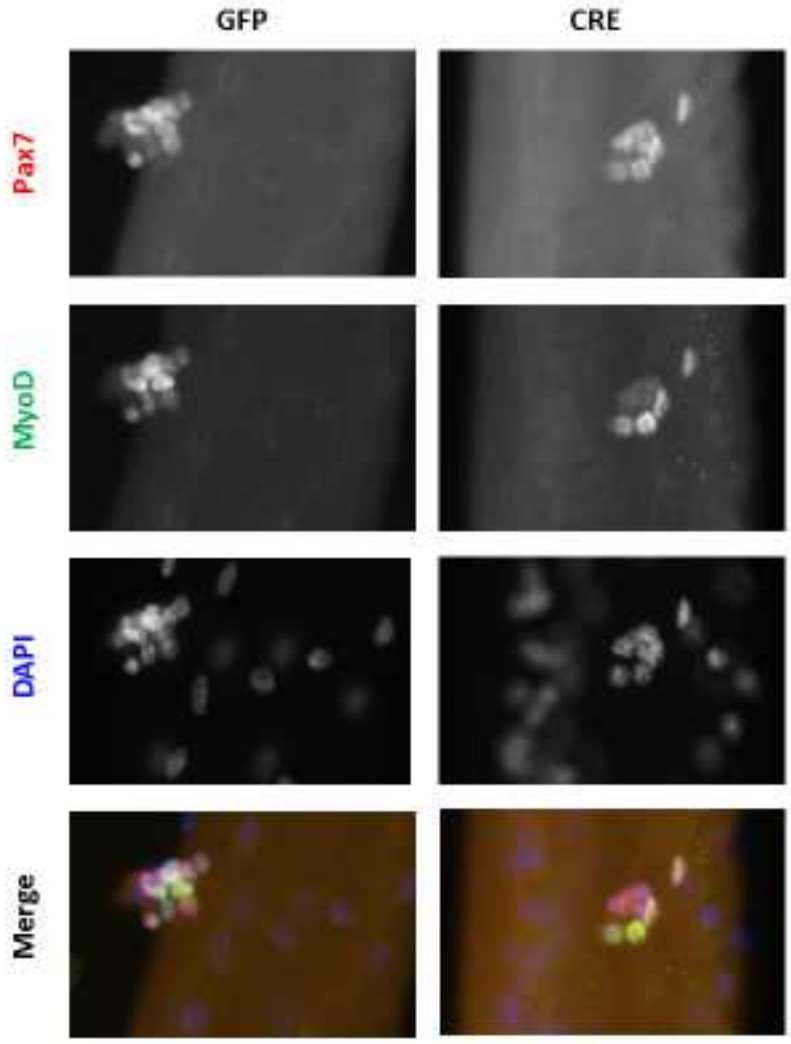

Figure 2.4 A Different Subpopulations in Satellite Cell Clusters Are Going through Self-renewal, Proliferation and Differentiation.
Muscle fibers from *Rosa26*^{NICD}*/MDX* mice were cultured for 48h and infected by Adenovirus (GFP and Cre) for 24 more hours. The cell clusters were labelled with Pax7 (red) and MyoD (green). Quiescent, proliferating and differentiating cells were labeled by Pax7+MyoD-, Pax7+MyoD+, and Pax7-MyoD staining, respectively. Nuclei were counterstained with DAPI (blue).

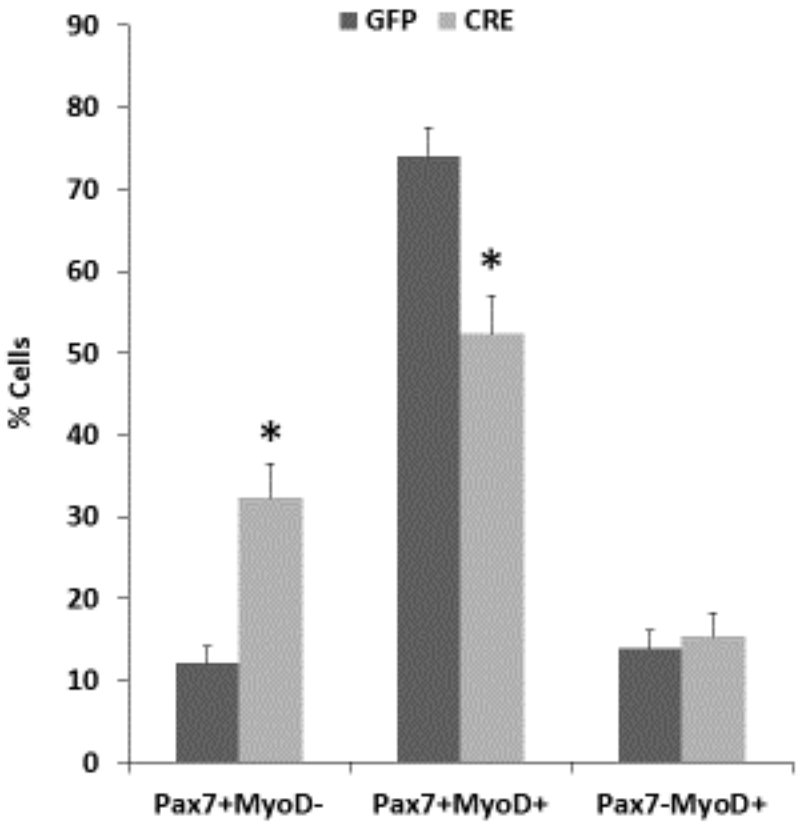

Figure 2.4 B Notch Activation Promotes Self-renewal of Satellite Cells. Percentages of subpopulations of cells in one cluster were quantified based on Pax7 and MyoD staining. n=3 independent experiment with more than 20 clusters analyzed in each experiment. Error bars represent s.e.m. *P<0.05.

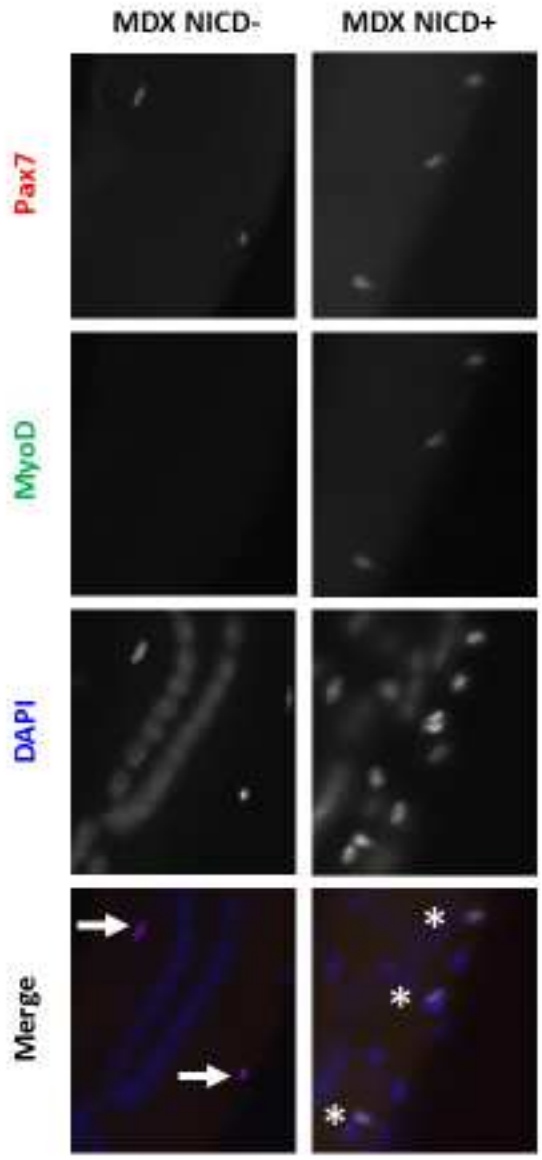

Figure 2.4 C Tamoxifen Activates the Notch Pathway in the Satellite Cells of MDX/NICD+ Mice.
After tamoxifen injections, muscle fibers from MDX/NICD- ($Pax7^{CreER/+}$/MDX) and MDX/NICD+ ($Pax7^{CreER/+}$/$Rosa26^{NICD}$/MDX) mice were isolated and labeled with Pax7 (red) and GFP (green). Nuclei were counterstained with DAPI (blue). GFP signal is turned on by Cre in the $Rosa26^{NICD\text{-}ires\text{-}nGFP}$ mice (called $Rosa26^{NICD}$ here).

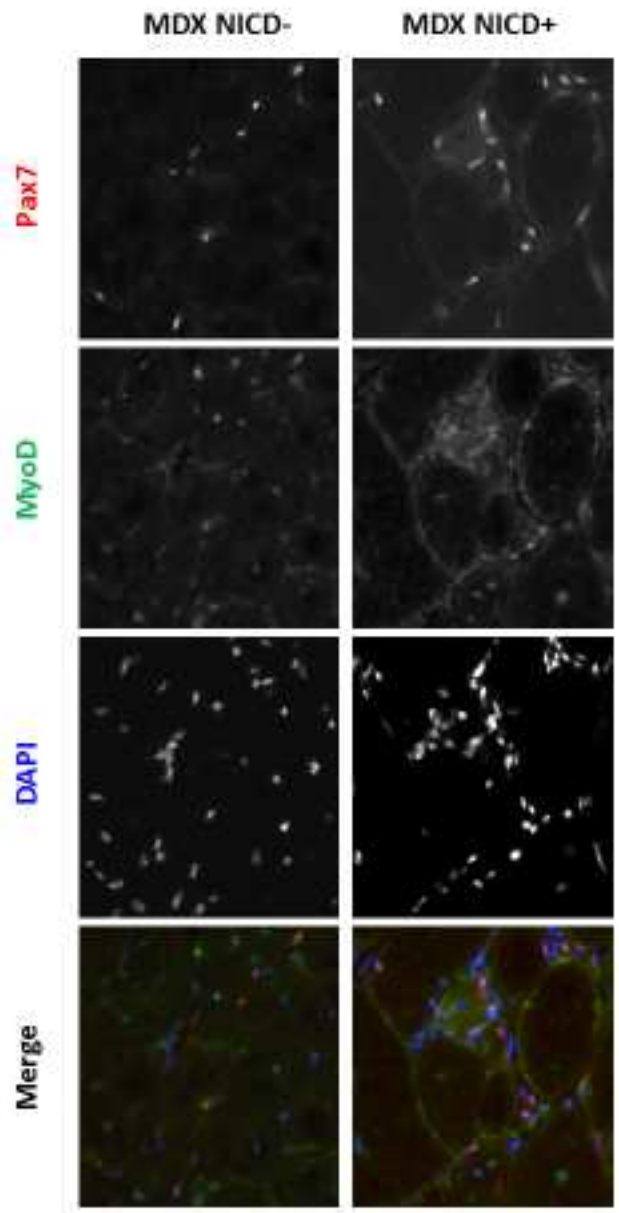

Figure 2.4 D Different Subpopulations of Satellite Cells Are Going through Self-renewal, Proliferation and Differentiation.
Two-month-old MDX/NICD^{-} and MDX/NICD^{+} mice were injected intraperitoneally with Tamoxifen for 5 consecutive days. Then TA muscles were injected with CTX and samples were collected 7 days after CTX injection. TA cross sections were stained with Pax7 (red) and MyoD (green). Nuclei were counterstained with DAPI (blue).

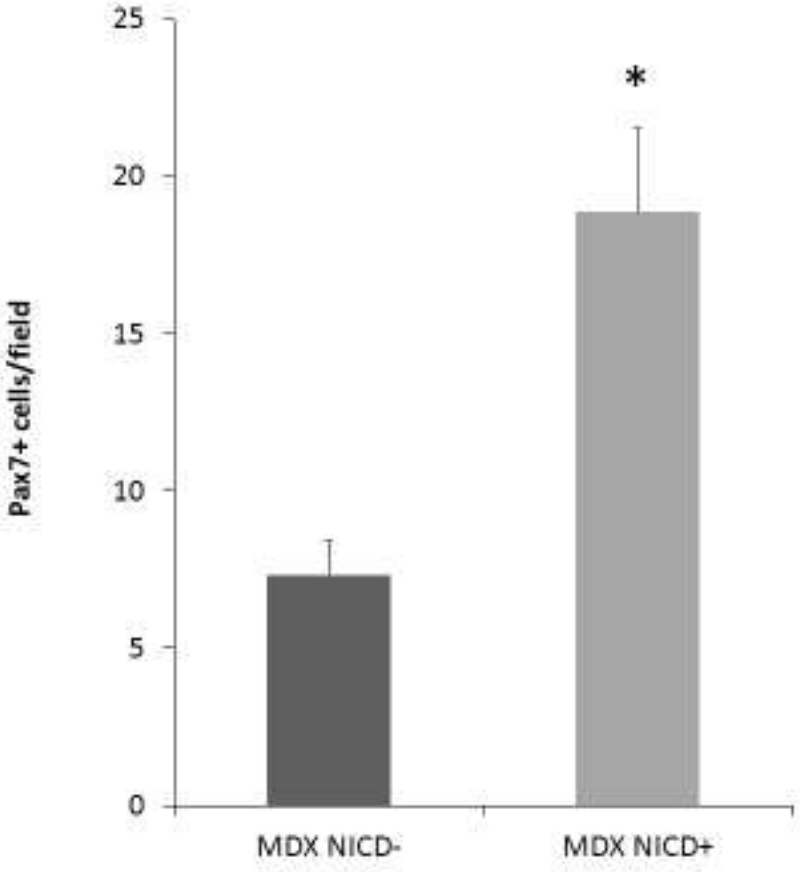

Figure 2.4 E The Activation of Notch Increases Satellite Cell Number in MDX Muscles.
After staining with Pax7, the number of satellite cells per field was quantified. n=3 mice with more than five fields were analyzed in each mouse. Error bars represent s.e.m. *P<0.05.

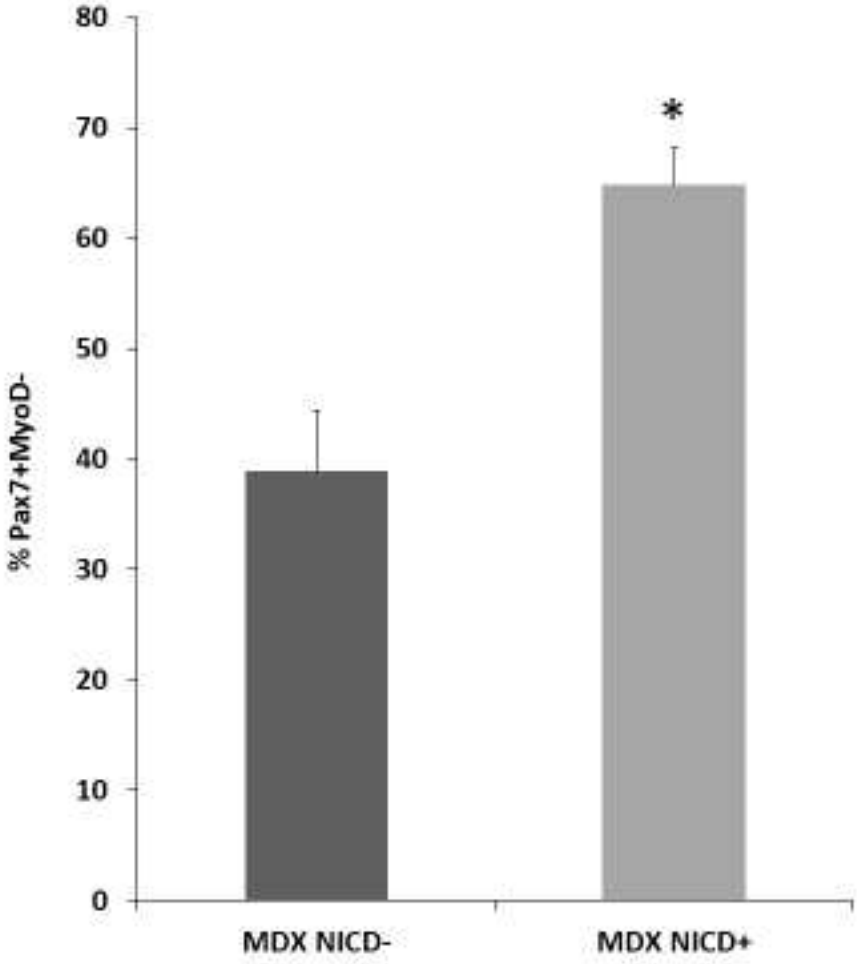

Figure 2.4 F The Activation of Notch in Satellite Cells Increases Self-renewal in MDX Muscles.
After staining with Pax7 and MyoD, the ratios of self-renewal satellite cells (Pax7+/MyoD-) per field were quantified. n=3 mice with more than five fields were analyzed in each mouse. Error bars represent s.e.m. *P<0.05.

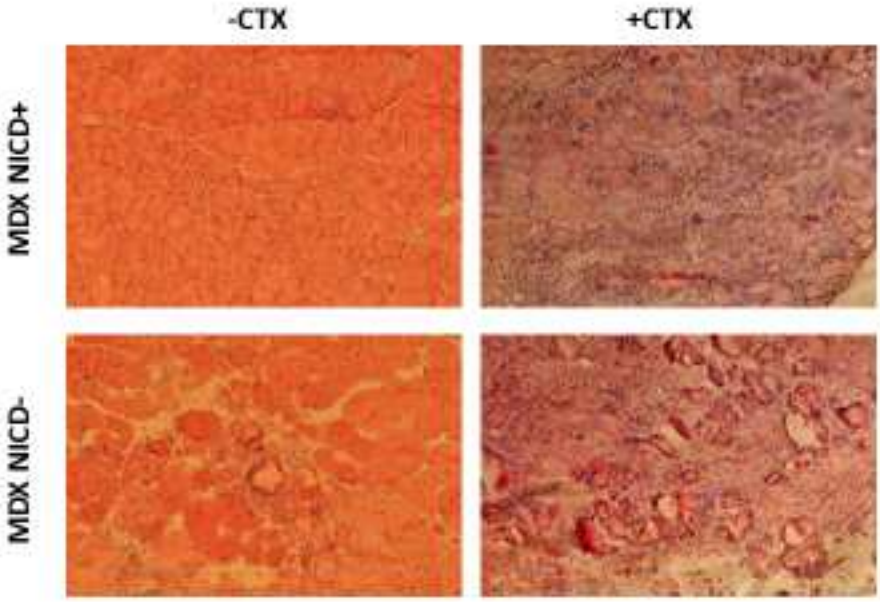

Figure 2.4 G The Activation of Notch in Satellite Cells Deteriorates Muscle Regeneration in MDX Mice.
Two-month-old MDX/NICD^{-} and MDX/NICD^{+} mice were injected intraperitoneally with Tamoxifen for 5 consecutive days. Then TA muscles were injected with CTX and samples were collected 7 days after CTX injection. TA cross sections were subjected to H&E staining.

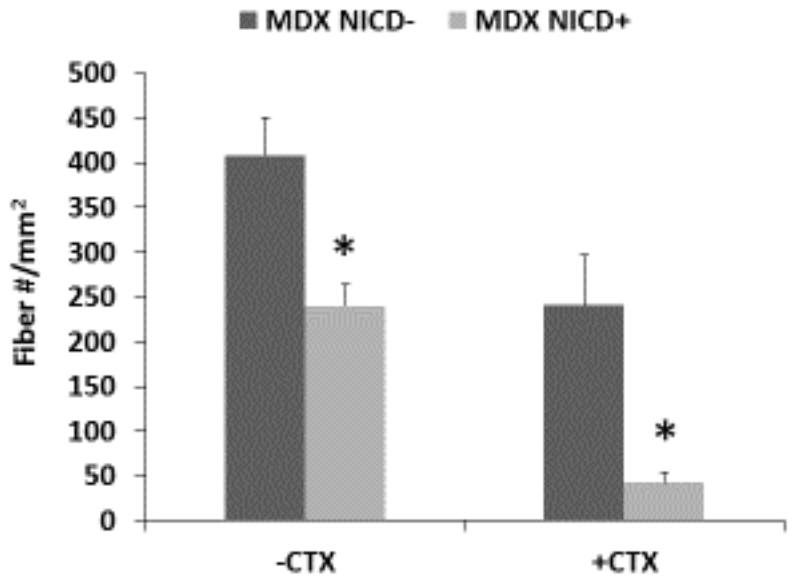

Figure 2.4 H The Activation of Notch in MDX Satellite Cells Leads to Less Myofiber Formation in MDX Muscle.
The number of myofibers (>500 μm^2) per field (1 mm^2) was calculated. n=3 mice with more than five fields were analyzed in each mouse. Error bars represent s.e.m. *P<0.05.

CHAPTER 3. CHARACTERIZATION OF THE ROLES OF BEX1 IN MYOGENESIS

3.1 Abstract

Muscle regeneration is a complex but precisely orchestrated process involving spatiotemporal regulation of genes critical for myogenesis. Brain expressed x-linked gene 1 (Bex1) has recently been reported to be involved in muscle regeneration. How Bex1 regulates myogenesis, however, is completely unknown. To address this question, I first characterized the expression pattern of Bex1 in muscle development and regeneration. Bex1 is expressed following myogenic differentiation during embryonic muscle development. In adult muscle regeneration, Bex1 is highly induced in the early stage of regeneration and gradually disappears with muscle recovery. Consistently, in cultured myoblasts, Bex1 was not expressed at the proliferation stage but highly expressed upon induction of myogenic differentiation. Interestingly, Bex1 protein was mainly localized to the nucleus of newly differentiated myotubes, suggesting a role of Bex1 in regulating muscle gene expression. Using gain- and loss-of-function studies in cultured myoblasts, I observed that overexpression of Bex1 did not affect myogenic differentiation but dramatically promoted myoblast fusion.

Conversely, Bex1 knockout myotubes exhibited fusion defects. These results elucidate a novel role of Bex1 in myogenesis through regulating myoblast fusion.

Keywords: Bex1, muscle regeneration, myoblast fusion

3.2 Introduction

Under normal conditions, mammalian adult skeletal muscle is relatively stable with little nuclei turnover, no more than 1-2 percent per week (Schmalbruch and Lewis, 2000). However, skeletal muscle is susceptible to a variety of injuries. Upon injury, skeletal muscle has the outstanding capacity to initiate a rapid and extensive repair process, known as muscle regeneration, to prevent further muscle loss. Of note, muscle stem cells, satellite cells, play indispensable roles in muscle regeneration (Sambasivan et al., 2011; von Maltzahn et al., 2013). In the early stage of muscle regeneration, satellite cells are activated from quiescence and proliferate as myoblasts to generate a sufficient number of cells. Subsequently, a majority of the proliferating myoblasts withdraw from the cell cycle and fuse to the injury sites to repair muscle damage. The mechanism involved in muscle regeneration consists of multiple signaling pathways. Unraveling the regulatory network behind muscle regeneration has drawn intense research attention in the field.

Myoblast fusion is a crucial cellular process contributing to muscle regeneration as well as muscle growth and development. Myoblast fusion is characterized by cell attraction, migration, adhesion, and alignment followed by the membrane rearrangement and finally resolution (Doberstein et al., 1997). The fusion process occurs at two phases. The primary stage leads to the formation of nascent myotubes with few nuclei from myoblast-myoblast fusion. The secondary stage results in the formation of large syncytia with increased nuclear number and augmented myotube size from myoblast fusion with nascent myotubes

(Horsley and Pavlath, 2004). Many progresses have been made in unraveling signaling pathways behind myoblast fusion in Drosophila, which occurs between two genetically different cell subpopulations of founder and fusion-competent myoblasts (Abmayr and Pavlath, 2012). Of note, ELMO-Myoblast city-Rac pathway has been determined to play essential roles in myoblast fusion (Duan et al., 2012; Geisbrecht et al., 2008; Rushton et al., 1995). Intriguingly, this signaling pathway is well conserved between Drosophila and vertebrates. It has been reported that ELMO-DOCK1 (ortholog of Myoblast city)-Rac also coordinately control the myoblast fusion in mice (Laurin et al., 2008). Furthermore, the ELMO-DOCK1-Rac pathway is under the control of brain-specific angiogenesis inhibitor (BAI) family members, including BAI1 and BAI3, both of which have been corroborated to promote myoblast fusion (Hamoud et al., 2014; Hochreiter-Hufford et al., 2013). Nevertheless, the signaling pathways controlling myoblast fusion in vertebrates remains largely elusive.

The relevance of Brain Expressed X-linked gene 1 (Bex1) in muscle regeneration was discovered in a microarray aimed to identify a panel of genes with altered expression in cardiotoxin (CTX) induced muscle injury, a well-established model for muscle regeneration (Goetsch et al., 2003; Yan et al., 2003). It was also supported by the observation that the expression level of Bex1 was dysregulated in MDX mice (Turk et al., 2005), which undergo progressive muscle degeneration and regeneration. Bex1 knockout mice displayed altered muscle regeneration, indicated by prolonged cell proliferation and delayed cell

differentiation (Koo et al., 2007). However, the detailed mechanism of how Bex1 is involved in muscle regeneration has been lacking.

Bex1 belongs to a small growing family including six members with high homology in gene sequences and structures but distinct in the expression pattern and subcellular localization (Alvarez et al., 2005). Until now, the functions of Bex1 have been largely unknown. Bex1 has been identified as a neuron regeneration gene, as Bex1 knockout mice are deficient in axon regeneration after sciatic-nerve injury(Khazaei et al., 2010). In addition, Bex1 levels are cell-cycle dependent in PC12 neuron cells, with the lowest expression level in G1 phase and the highest level in S phase. Moreover, down-regulation of Bex1 is necessary for neuron cells to exit the cell cycle, as overexpression of Bex1 results in sustained cell proliferation even under growth arrest conditions. Further studies have confirmed that Bex1 regulates cell cycle by interacting with p75 neurotrophin receptor (p75NTR) to regulate the neurotrophin signaling pathway (Vilar et al., 2006). Besides its roles in the nervous system, Bex1 has been determined as a candidate tumor suppressor gene because its inactivation is associated with the development of various types of tumors (Foltz et al., 2006; Karakoula et al., 2014; Lee et al., 2013).

In this study, I observed that Bex1 is expressed after the induction of myogenic differentiation and undergoes nuclear-cytoplasmic trafficking during embryonic muscle development. I also characterized that Bex1 is temporally induced during muscle regeneration. Consistently, I demonstrated that Bex1 is exclusively expressed in differentiated myoblasts *in vitro*. By gain-of-function

study, I determined that Bex1 promotes myoblast-myotube fusion without affecting myogenic differentiation *per se*. Conversely, primary myoblasts from Bex1 knockout mice are defective in fusion, although Bex1 knockout mice can regenerate their injured muscles normally. These data suggested that Bex1 is actively involved in the process of myogenesis by regulating myoblast fusion.

3.3 Materials and Methods

3.3.1 Animals

All procedures involving animal maintenance and experimental use were performed based on the instructions established by Purdue University's Animal Care and Use Committee. Bex1 KO mice were provided by Prof. Frank L. Margolis (Koo et al., 2007). Bex1 heterozygous mice were bred to generate Bex1 null and wild type littermates used as control in the experiments. The PCR genotyping was done as previously described (Koo et al., 2007).

3.3.2 Muscle Injury and Regeneration

Muscle regeneration was induced by intramuscular injection of Cardiotoxin (CTX; Sigma-Aldrich, St. Louis, MO). Mice were anesthetized with a ketamine-xylazine cocktail, then 50μl of 10 mM CTX was injected into the Tibialis Anterior (TA) muscle. Muscles were harvested at day 5 and 14 post injection for histological studies. Age- and gender-matched control mice were used for each experiment.

3.3.3 Culture of skeletal muscle derived primary cells

Primary cells were isolated from limb skeletal muscles of 2-month old mice. Muscles were minced and digested with a cocktail of type I collagenase and dispase B mixture (Roche Applied Science) and subsequently cultured in growth media (F-10 Ham's medium supplemented with 20% fetal bovine serum, 4 ng/mL basic fibroblast growth factor, and 1% penicillin-streptomycin) on collagen-coated dishes. Upon confluence, cells were differentiated in myogenic differentiation media (DMEM supplemented with 2% horse serum and 1% penicillin-streptomycin).

3.3.4 Cryosection

Fresh muscles were embedded in optimal cutting temperature (OCT) compound (Sakura Finetek) and immediately frozen in dry ice-cooled isopentane. Muscle blocks were cut by 10μm with a Leica CM 1850 cryostat instrument. The sections were placed on Superfrost Plus glass slides (Electron Microscopy Sciences).

3.3.5 Immunostaining and Image Capture

Muscle tissue sections and cell cultures were first fixed in 4% PFA solution and blocked in the blocking buffer containing PBS, 5% goat serum, 2% bovine serum albumin, 0.2% Triton X-100 and 0.1% sodium azide for 1 hour. Next, the sections or cell cultures were incubated with primary antibodies diluted in blocking buffer overnight at 4℃, then incubated with secondary antibodies and 4′,6-diamidino-2-phenylindole (DAPI) diluted in PBS for 30 minutes at room

temperature, and finally mounted with Dako fluorescent mounting media (Glostrup, Denmark). Fluorescent pictures were taken with a Coolsnap HQ CCD camera (Photometrics, USA) driven by IP Lab software (Scanalytics, USA) in a Leica DMI 6000B fluorescent microscope (Mannheim, Germany). As the analysis of the immunofluorescence was qualitative, identical image handling and fluorescence scoring criteria were applied in all the experiments.

3.3.6 Recombinant Adenovirus Construction and Infection

Recombinant adenoviruses expressing Bex1 were constructed using the Adeasy system, including the adenoviral plasmid (pAdEasy-1) and the shuttle vector (pAdTrack-CMV), both of which were kindly provided by Prof. Yongxu Wang. The sequence of Bex1 was PCR amplified and cloned into the pAdTrack-CMV vector. The recombination with the Adeasy1 plasmid and transfection to HEK293 cells as well as amplification of the recombinant adenovirus were performed as previously described (He et al., 1998). For infection experiments, myoblasts were grown to 80% confluence and infected with virus for 48h at 37℃.

3.3.7 Quantitative Real-time Polymerase Chain Reaction

RNA was extracted and purified from muscles or cell cultures using Trizol, followed by the digest with Turbo DNase (Ambion). Random hexamer primers were used for the reverse transcription from RNA to cDNA. qPCR was performed with a Light Cycler 480 machine (Roche). 18s was used as housekeeping gene for normalization. For qPCR result analysis, $2^{-\Delta\Delta ct}$ method was applied to calculate the fold change.

3.3.8 Protein Extraction and Western Blots Analysis

Total protein was extracted from muscles or cells using RIPA buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS). Protein concentrations were measured by Pierce BCA protein assay reagent (Pierce Biotechnology, Rockford, IL, USA). Proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF membranes (Millipore Corp., Billerica, MA), and probed with specific antibodies (Bex1, 1:5000 dilution, provided by Prof. Frank L. Margolis; Pax7, 1:10 culture supernatant, DSHB, U Iowa; myogenin, 1:1000 dilution, Santa Cruz Biotechnology; GAPDH, 1:1000 dilution, Santa Cruz Biotechnology), then detected by chemiluminescence with FluorChem™ R System (ProteinSimple, Santa Clara, CA, USA).

3.3.9 Statistical Analysis

Data are displayed with mean ± s.e.m. P-values were calculated by two-tailed Student's t-test. P-values <0.05 were considered to be statistically significant.

3.4 Results

3.4.1 Bex1 Expression during Muscle Development and Regeneration

Previous studies demonstrated that Bex1 is predominantly expressed in the central nervous system. Besides, it is widely expressed in various tissues with high levels in the liver but less abundance in skeletal muscles (Alvarez et al., 2005). However, it has been documented that Bex1 is highly expressed in

somatic mesenchyme during muscle development (Vilar et al., 2006). Here I sought to further characterize the *in vivo* expression pattern of Bex1 during skeletal muscle development and regeneration.

In the mouse embryonic muscle development, Pax7 and myogenin have been regarded as markers of muscle progenitor cells and differentiated myocytes, respectively (Cheng et al., 1992; Relaix et al., 2006; Venuti et al., 1995). Using a Bex1 antibody, I detected immunofluorescence in developing somites at embryonic day (E) 10.5. Co-localization analysis indicates that Bex1 is absent in the Pax7-expressing cells but co-localizes with a subpopulation of myogenin-expressing myogenic cells (**Figure 3.1A**), indicating that Bex1 is only expressed in differentiating or differentiated muscle cells. Interestingly, analysis of intracellular localization indicates that Bex1 is located in the cytoplasm at E10.5 but in the nucleus at E12.5 (**Figure 3.1B**). The late expression of Bex1 in myogenin-expressing cells suggests that Bex1 might play a role in myogenic differentiation.

I also examined the expression pattern of Bex1 in postnatal muscle regeneration. Before muscle injury, Bex1 was barely detectable in rested muscles (**Figure 3.1C**), suggesting that Bex1 is not required for maintaining normal muscle function. During muscle regeneration, Bex1 was highly induced in the cytoplasm of regenerating muscles at Day 5 but its expression disappeared at Day 14 (**Figure 3.1C**), indicative of a spatiotemporal expression pattern. In agreement with this observation, Bex1 transcript level was detected to peak at Day 5 post CTX injury (**Figure 3.1D**), when myoblasts have been extensively

expanded and are fusion competent (Robertson et al., 1990). Then Bex1 expression gradually disappeared afterwards until nearly undetectable at Day 14 (**Figure 3.1D**), when the muscle regeneration is largely completed. Altogether, these data indicate that Bex1 is actively involved in embryonic and postnatal myogenesis.

3.4.2 Bex1 is Exclusively Expressed in Differentiated Myoblasts *in vitro*.

In order to better understand the role of Bex1 in the myogenesis, I also examined the expression pattern of Bex1 in cultured primary myoblasts. In agreement with the expression pattern of Bex1 in embryonic muscle development, Bex1 is absent in proliferating myoblasts while its expression is highly induced by differentiation (**Figure 3.2A**). Consistently, the expression levels of Bex1 mRNA and protein were increased in differentiated myocytes compared to undifferentiated myblasts (**Figure 3.2B** and **2C**). In addition, Bex1 expression is limited to the nuclei of differentiated myotubes (**Figure 3.2A**). Moreover, single fiber culture experiment was performed to investigate the Bex1 expression pattern in myoblasts attached to the host fiber. After culture for 72 hours, Bex1 is not expressed in Pax7+ (**Figure 3.2D**) or KI67+ cells (**Figure 3.2E**), supporting the notion that Bex1 is not expressed until differentiation. While the expression of Bex1 emerged in the myogenin+ cells, mainly in the nucleus but also in the cytoplasm (**Figure 3.2F**), confirming that Bex1 is induced upon myogenic differentiation. Notably, given the lack of Bex1 localization in the nucleus of mature myotubes, these data suggest that Bex1 undergoes a nucleus to cytoplasm transition during maturation of nascent myotubes. Moreover, the

exclusive expression pattern of Bex1 in nascent myotubes prompted us to explore the roles of Bex1 in myogenic differentiation.

3.4.3 Bex1 does not Regulate Cell Cycle Withdrawal in Myogenic Cells.

Prior studies demonstrated that overexpression of Bex1 in neuron cells renders resistance to NGF- or serum withdrawal-induced growth arrest, whereas Bex1 knockdown led to cell cycle exit and premature differentiation. Furthermore, Bex1 was determined to interact with p75NTR to regulate cell cycle withdrawal (Vilar et al., 2006). Given that Bex1 is cell-cycle-regulated with the lowest in G1 phase and highest in S phase in neuron cells, I first examined whether Bex1 could be accumulated in the specific phase of cell cycle. However, I observed that Bex1 was undetectable with treatment of mimosine, thymidine, and nocodazole (arresting cells in the G1, S, G2/M phase, respectively) (**Figure 3.3A**), which suggests that Bex1 is not dependent on cell cycle progression. Moreover, I performed FACS analysis on undifferentiated myoblast infected by adenovirus expressing GFP and Bex1. The ectopic expression of Bex1 in proliferating myoblasts failed to promote cell cycle withdrawal, indicated by the comparable distribution of cells in G1, S, G2/M phase (**Figure 3.3B**). Quantification of different cell groups confirmed that Bex1 overexpression did not affect the percentages of myoblast population in G1, S and G2/M phases. Collectively, these data demonstrated that Bex1 is not involved in myoblast cell cycle regulation.

3.4.4 Bex1 Promotes Myoblast-Myotube Fusion *in vitro.*

I further investigated the mechanism of how Bex1 regulates myogenic differentiation. It was reported that Bex1 can directly interact with Calmodulin (CaM), the ubiquitous Ca^{2+} -binding protein, in a Ca^{2+}-dependent manner *in vitro* (Koo et al., 2007). CaM can activate multiple CaM-dependent enzymes, such as Calcineurin, to mediate the regulatory effect of Ca^{2+} signaling pathway, which plays indispensable roles in the fusion of myoblast into multinucleated myotubes (Bar-Sagi and Prives, 1983; Bijlenga et al., 2000; Constantin et al., 1996; Shin et al., 1996).

To test whether Bex1 is involved in the myoblast fusion, I performed gain-of-function study on primary myoblasts. Bex1 mRNA transcript and protein were significantly increased after infection (**Figure 3.4A** and **3.4G**). After differentiation for 24hs, both GFP control and Bex1 overexpressed myoblasts started to form nascent myotubes with few nuclei and are indistinguishable in myofiber size (**Figure 3.4B**), confirmed by comparable differentiation index and fusion index (**Figure 3.4C** and **3.4D**). This suggested that Bex1 did not regulate the initial formation of myotubes. Interestingly, after differentiation for 96hs, Bex1 overexpression caused a dramatic accumulation of nuclei in mature myotubes (**Figure 3.4E**). The percentages of myofibers with more than 5 nuclei was markedly increased by Bex1 overexpression (13.56% vs 26.70%) (**Figure 3.4F**), suggesting that Bex1 positively regulates myoblast fusion with myotubes. However, the expression of myogenic differentiation markers, myogenin and myosin heavy chain (MHC), were comparable between two groups (**Figure 3.4G**),

indicating that Bex1 functions specifically through promoting myoblast fusion rather than regulating myogenic differentiation per se. Meanwhile, I also electroporated plasmid encoding Bex1 to C2C12 myoblasts to investigate the roles of Bex1 in myoblast fusion (**Figure 3.4H**). Consistently, the average number of myonuclei inside Bex1 overexpressed myotubes increased dramatically (4.7 vs 9.4) (**Figure 3.4I**), supporting the notion that Bex1 could promote myoblast fusion. Altogether, these data suggest that Bex1 promotes myoblast fusion without affecting myogenic differentiation.

3.4.5 Bex1 Knockout Myoblasts are Defective in Myoblast-myotube Fusion *in vitro*.

To confirm definitively that Bex1 promotes myoblast fusion, I bred Bex1 heterozygous mice to obtain the knockout mice. I administrated CTX to injure TA muscles and allowed muscle regeneration for 14 days. The Bex1 knockout muscles did not exhibit obvious regeneration defects at day 5 and day 14. Through laminin staining, I quantified the size of regenerated myofibers, a direct indicator of myoblast fusion. Bex1 knockout mice did not exhibit obvious fusion defects (**Figure 3.5A**), indicated by a cohort of indistinguishable fiber diameter classes (**Figure 3.5B**).

By culturing primary myoblast from Bex1 knockout mice, I performed a loss-of-function study to investigate the effects of loss of Bex1 on myoblast fusion *in vitro*. The primary myoblasts from Bex1 knockout mice were normal in terms of proliferation capacity like WT myoblasts, indicated by normal growth curve and comparable levels of Ki67, the well-established marker for cell proliferation (data

not shown). After differentiation for 24hs, Bex1 knockout myoblasts formed nascent myotubes normally, which is comparable with WT control in terms of nuclei number in the myotubes and fiber size (data not shown). However, after differentiation for 96hs, WT control myoblasts formed large myofibers with multiple nuclei inside whereas Bex1 knockout myoblasts failed to enlarge fiber size and accumulate myonuclei efficiently (**Figure 3.5C**). The percentages of myofibers with more than 5 nuclei was significantly reduced (43.43% vs 24.45%) (**Figure 3.5D**), suggesting that Bex1 knockout led to significant defects of myoblast fusion with myotubes. Besides, in accordance with the notion that Bex1 overexpression did not affect myoblast differentiation, knocking-out Bex1 did not impair the myoblast differentiation *per se*, indicated by the comparable expression levels of myogenic differentiation markers, myogenin and MHC (**Figure 3.5E**). Altogether, these data provided compelling evidence that Bex1 promotes myoblast-myotube fusion.

3.5 Discussions

In this study, I identified that Bex1 is expressed following myogenic differentiation during embryonic muscle development as well as temporarily induced during muscle regeneration. I also determined that Bex1 is specifically expressed in differentiated myocytes *in vitro*. Of note, Bex1 undergoes translocation from the cytoplasm to the nucleus as differentiation proceeds. I verified that Bex1 can promote myoblast-myotube fusion without affecting differentiation progression. Conversely, I demonstrated that Bex1 knockout caused impaired myoblast-myotube fusion. These results collectively elucidate

the role of Bex1 in myogenesis that Bex1 could promote myoblast-myotube fusion.

It has been well documented that Bex1 is abundantly expressed in neurons with a dynamic nucleocytoplasmic distribution pattern. This nucleocytoplasmic trafficking was associated with neuron growth factor (NGF) induced p75NTR signaling pathway (Lee et al., 2013). Here, I also observed the nucleocytoplasmic distribution pattern in myogenic cells. Bex1 was initially diffused in the cytoplasm but translocated to the nucleus in the late stage of differentiation. This nucleocytoplasmic trafficking could also be related to the p75NTR signaling pathway as p75NTR has been reported to have high expression levels in developing rat myoblasts as well as in rat and chicken muscles (Ernfors et al., 1988; Lomen-Hoerth and Shooter, 1995; Raivich et al., 1985; Raivich et al., 1987; Schecterson and Bothwell, 1992; Yamamoto et al., 1996). Another possible signaling pathway is the Ca^{2+} dependent signaling pathway, which plays indispensable roles in myogenic differentiation. It has been reported that Bex1 can directly interact with Calmodulin (CaM) (Koo et al., 2007), the ubiquitous Ca^{2+} -binding protein, which mediates the uptake of various nuclear proteins (Sweitzer and Hanover, 1996), such as NFAT isoforms. Future work can be focused on investigating which signaling pathway controls the nucleocytoplasmic trafficking of Bex1 in myogenesis.

Mammalian skeletal muscles are generated through the proliferation, differentiation and fusion of myoblasts into multinucleated myofibers. The spatiotemporal expression pattern of Bex1 suggests that Bex1 might be involved

in critical steps during myogenic differentiation. Previous studies demonstrated that Bex1 knockout mice posed prolonged proliferation and delayed differentiation kinetics compared to WT mice during muscle regeneration (Koo et al., 2007), indicating the defects of cell cycle withdrawal due to the loss of Bex1. However, our studies show that the myoblasts cultured from Bex1 knockout mice were not defective in proliferation *in vitro*. Also, Bex1 ectopic expression did not regulate cell cycle withdrawal and further myogenic differentiation *per se*. The observation of prolonged proliferation and delayed differentiation might be due to other cell types in that muscle regeneration involves proliferation of various types of cells, such as immune cells.

Furthermore, I verified that Bex1 could positively regulate myoblast fusion. Interestingly, the myoblast-myoblast fusion was not affected by either Bex1 overexpression or knock-out. However, the ectopic expression of Bex1 dramatically increased the myoblast-myotube fusion *in vitro* while Bex1 knockout myoblasts were defective in the process. Recently, it was reported that apoptotic cells can induce ELMO/DOCK1/Rac pathway through the cell surface protein BAI1 to enhance myoblast fusion (Hochreiter-Hufford et al., 2013). Besides, Bex3 has been identified to interact with p75NTR to mediate apoptosis pathway (Mukai et al., 2000). Given that Bex1 and Bex3 share the similar sequences implicated in p75NTR binding (Vilar et al., 2006), it is plausible to hypothesize that Bex1 could promote apoptosis during myogenic differentiation, which initiates the ELMO/DOCK1/Rac signaling pathway and mediates the fusion. However, during the muscle regeneration, Bex1 knockout mice did not exhibit

deficient myoblast fusion as well as impaired muscle regeneration. This might be attributable to the compensation effect of other Bex family members due to the loss of Bex1. Or, the massive degeneration overwhelmed the effect of loss of Bex1 on cell apoptosis, which further masked the defects of myoblast fusion. Generating Bex1 transgenic mice may provide additional clues to study the roles of Bex1 in muscle regeneration. Nevertheless, this study contributes to our understanding the cellular and molecular mechanism of myogenesis, especially the myoblast fusion. These data may have implication in the development of therapeutic strategies of muscle diseases.

3.6 Acknowledgements

The authors would like to thank Frank L. Margolis (University of Maryland) for providing the Bex1 knockout mice; Signe Hobaugh and Jun Wu for maintaining mouse colonies and lab duties; and other members of the Kuang lab for technical assistance and discussion.

3.7 Competing Interests Statement

The authors declare no conflict of interests.

3.8 Author Contributions

C. J. and J. W. designed and performed the experiments. C. J. and S. K. wrote the paper.

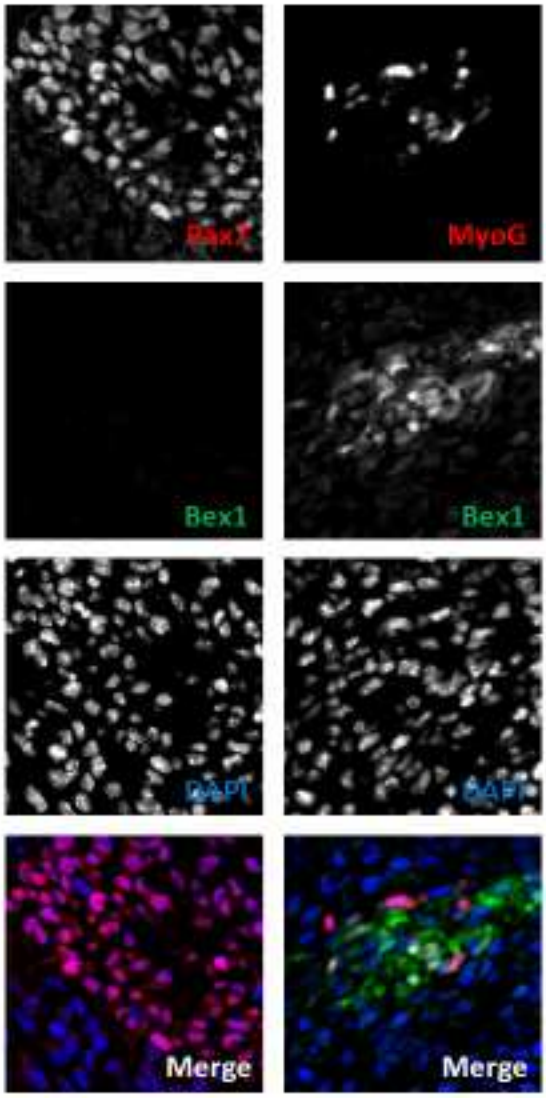

Figure 3.1 A Bex1 Is Expressed During Myogenic Differentiation *in vivo*. Embryos at E10.5 stage were sectioned and applied in the immunostaining with antibodies recognizing Bex1 (green) together with Pax7 (red, left panel) and myogenin (red, right panel), showing a concurrent expression pattern of Bex1 and myogenin. Nuclei were counterstained with DAPI (blue).

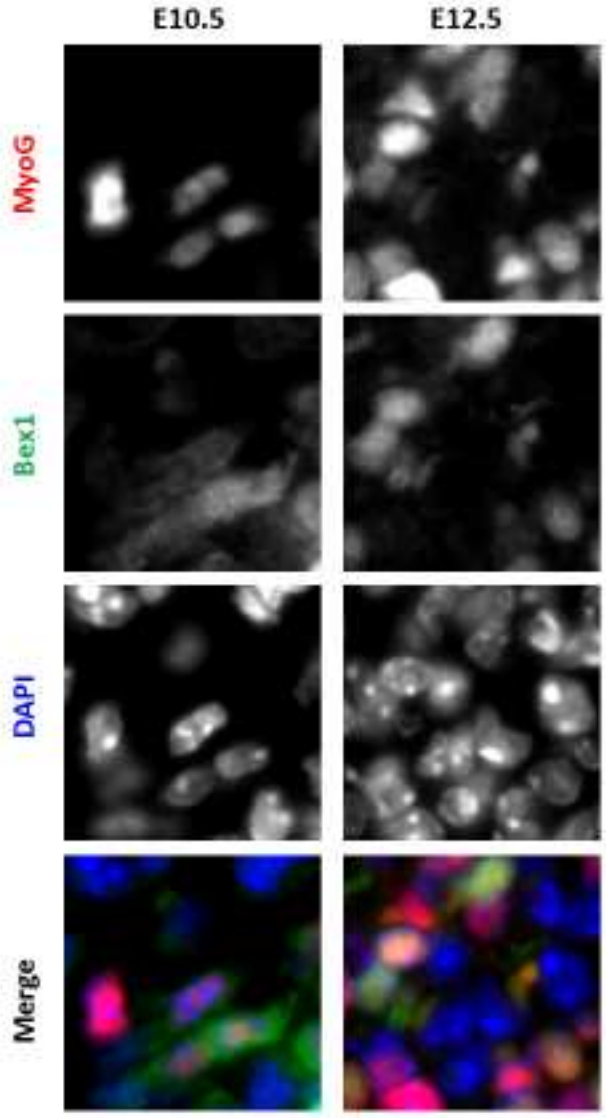

Figure 3.1 B Bex1 Translocates from Cytoplasm to Nucleus with Myoblast Differentiation *in vivo*.
Embryos at E10.5 (left panels) and E12.5 stage (right panels) were sectioned and applied in the immunostaining with antibodies recognizing myogenin (red) and Bex1 (green). Bex1 undergoes cytonucleotrafficking from cytoplasm at E10.5 to nucleus at E12.5. Nuclei were counterstained with DAPI (blue).

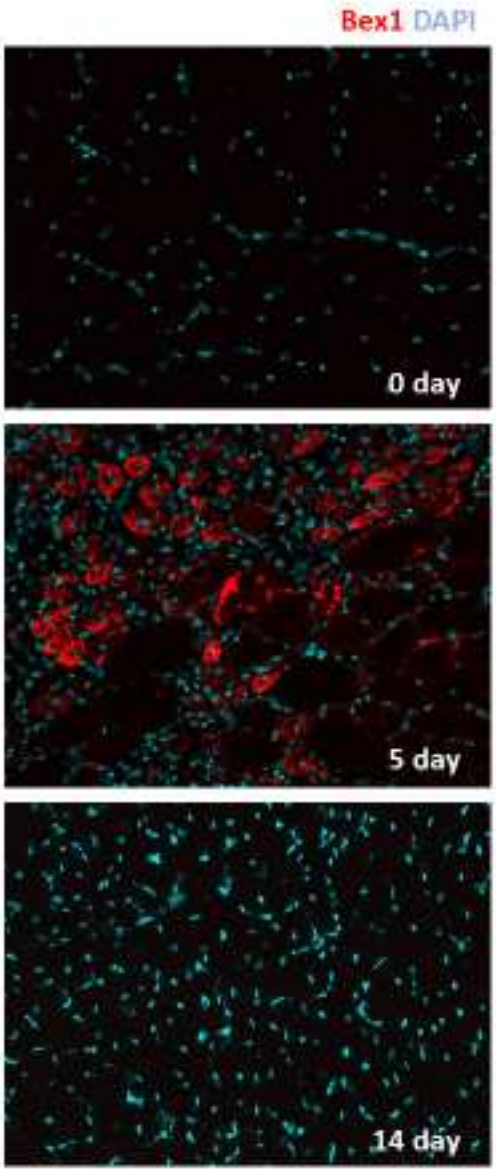

Figure 3.1 C Bex1 Is Temporarily Expressed during Muscle Regeneration. TA muscles were injected with CTX and samples were collected at indicated points after CTX injection. TA cross sections were stained with Bex1 (red). Nuclei were counterstained with DAPI (cyan).

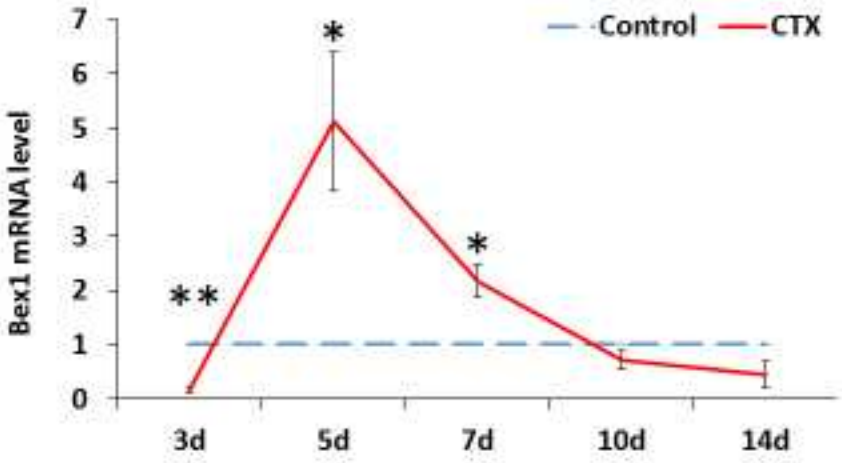

Figure 3.1 D Bex1 Is Highly Induced at Day 5 post Injury but Gradually Disappears afterwards.
TA muscles were injected with CTX and samples were collected at indicated points after CTX injection. RNA were extracted from muscles and compared between rested and injured muscles at different time points. The dot line indicates the expression level of Bex1 in rested muscles. N=3. Error bars represent s.e.m. *P<0.05 compared with rested muscles.

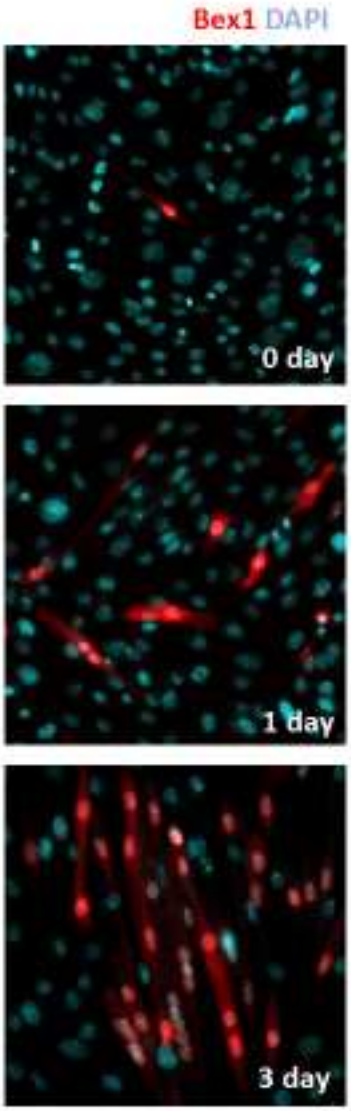

Figure 3.2 A Bex1 Is Exclusively Expressed in Differentiated Myocytes. Primary myoblasts were cultured and induced for differentiation. Cell culture at different days post differentiation induction was applied in the immunostaining with antibody recognizing Bex1 (red). Nuclei were counterstained with DAPI (cyan).

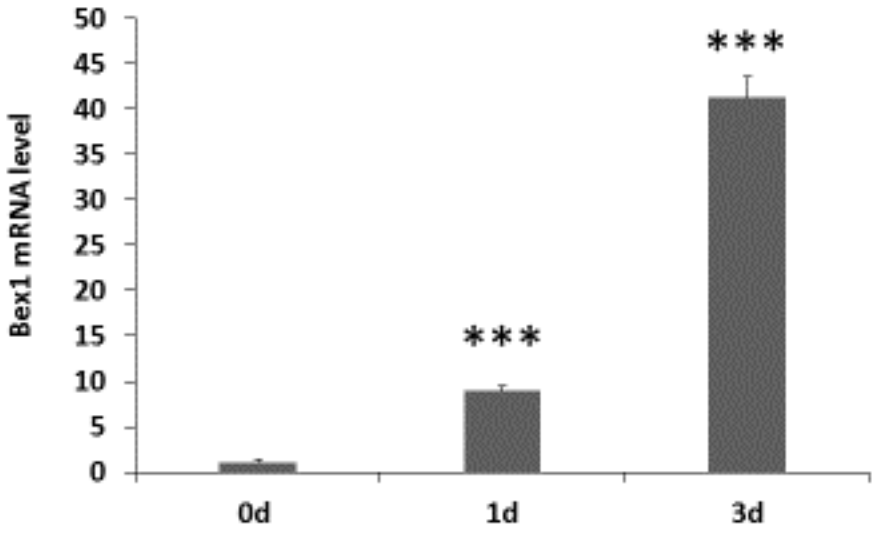

Figure 3.2 B The Expression Level of Bex1 Tanscript Is Increased with Differentiation.
Primary myoblasts were cultured and induced for differentiation. RNAs were extracted from cell culture at different days post differentiation induction. N=3 independent experiments. Error bars represent s.e.m. *P<0.05.

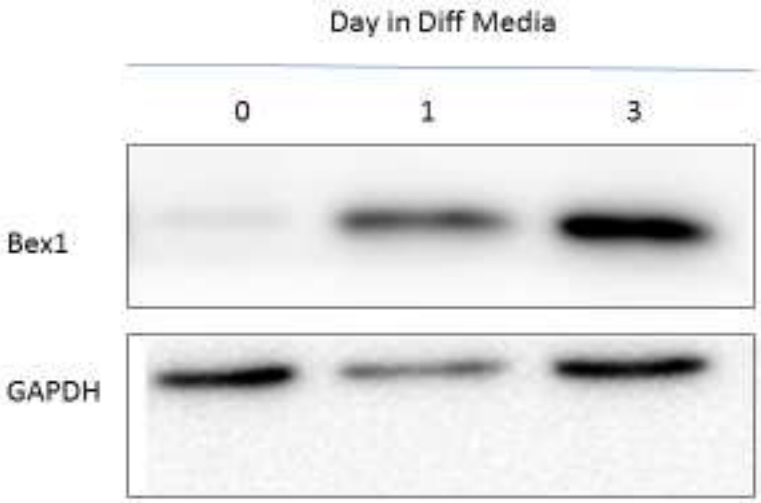

Figure 3.2 C The Expression Level of Bex1 Protein Is Increased with Differentiation.
Primary myoblasts were cultured and induced for differentiation. Proteins were extracted from cell culture at different days post differentiation induction. N=3 independent experiments.

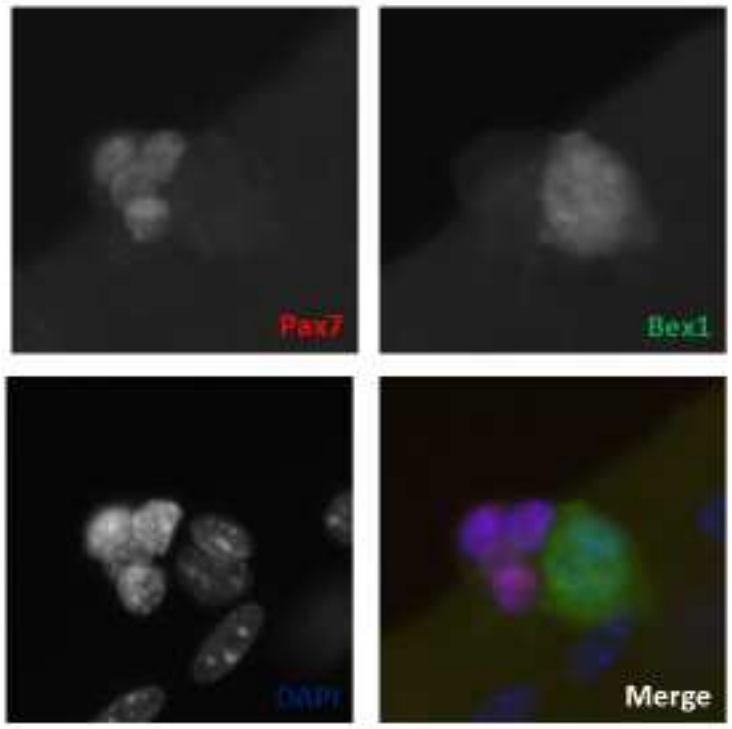

Figure 3.2 D Bex1 Is Not Expressed in Undifferentiated Myoblasts.
Single fibers were isolated and cultured for 72hs. Satellite cell clusters were formed and labeled with Bex1 (green) together with Pax7 (red, top). Nuclei were counterstained with DAPI (blue).

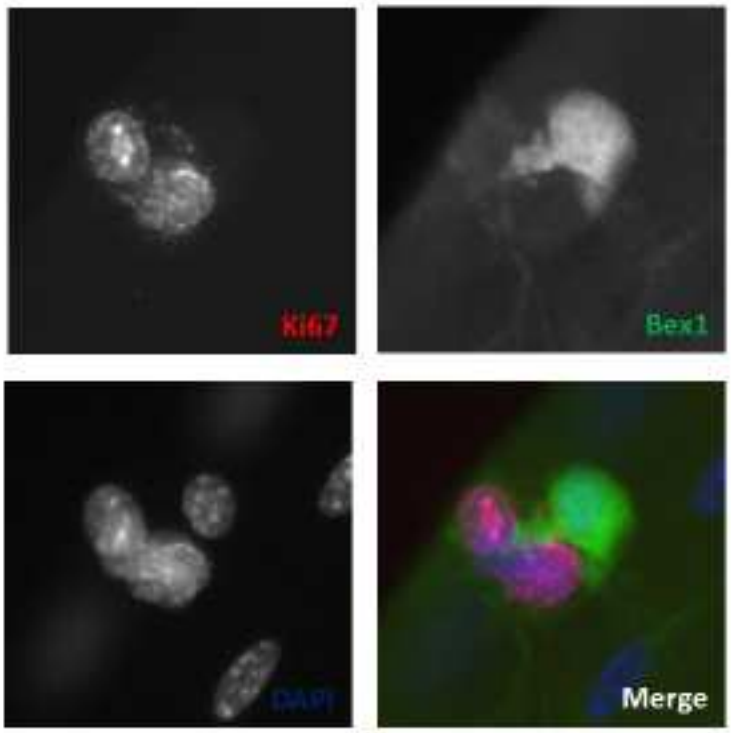

Figure 3.2 E Bex1 Is Not Expressed in Proliferating Myoblasts.
Single fibers were isolated and cultured for 72hs. Satellite cell clusters were formed and labeled with Bex1 (green) together with Ki67 (red, top). Nuclei were counterstained with DAPI (blue).

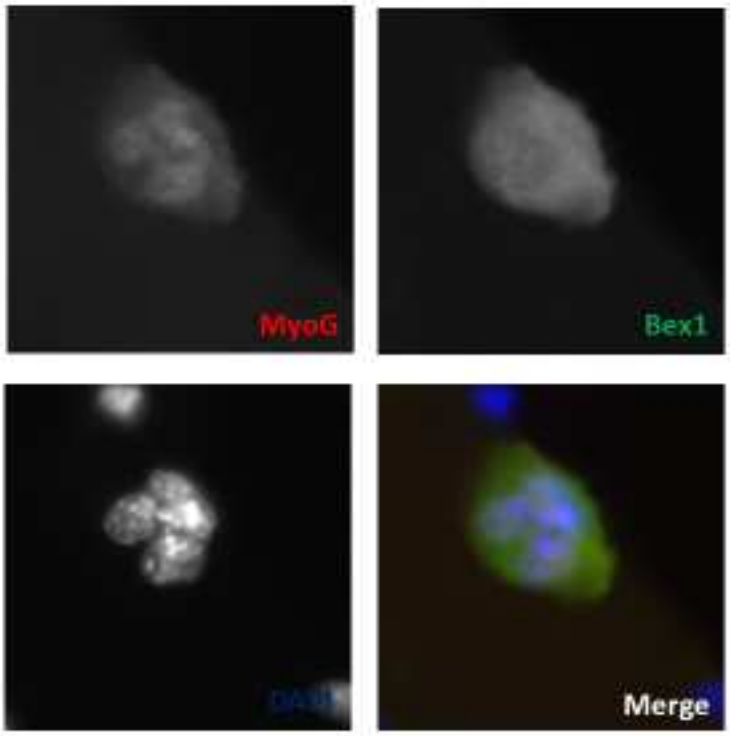

Figure 3.2 F Bex1 Is Expressed in Differentiated Myoblast.
Single fibers were isolated and cultured for 72hs. Satellite cell clusters were formed and labeled with Bex1 (green) together with myogenin (red, top). Nuclei were counterstained with DAPI (blue).

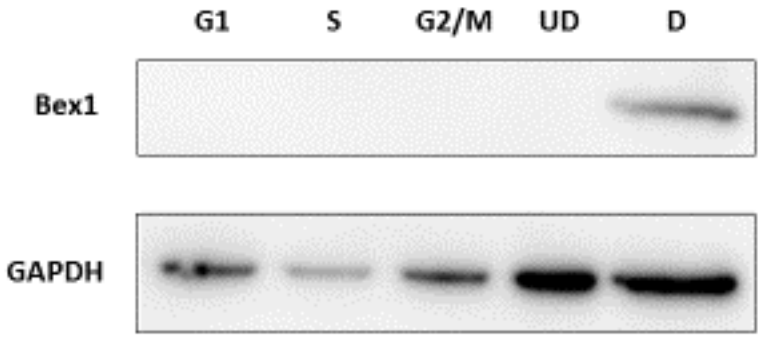

Figure 3.3 A Bex1 Is Not Cell-cycle-regulated.
Primary myoblasts were cultured and treated with mimosine, thymidine, and nocodazole, which can arrest cells at G1, S, and G2/M phases, respectively. UD, undifferentiated myoblast. D, differentiated myotubes. Proteins were collected and subjected to SDS page. N=3 independent experiments.

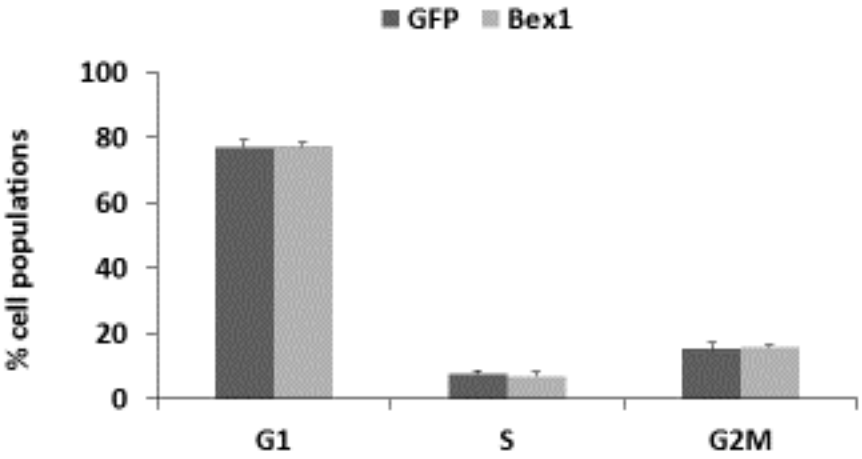

Figure 3.3 B The Ectopic Expression of Bex1 Does Not Promote Cell Cycle Withdrawal.
Primary myoblasts were infected with virus expressing GFP and Bex1 for 48hs. Cell population at different phases of cell cycle were separated by FACS. Quantification of ratio of cell population at G1, S, and G2/M phases. N=3 independent experiments.

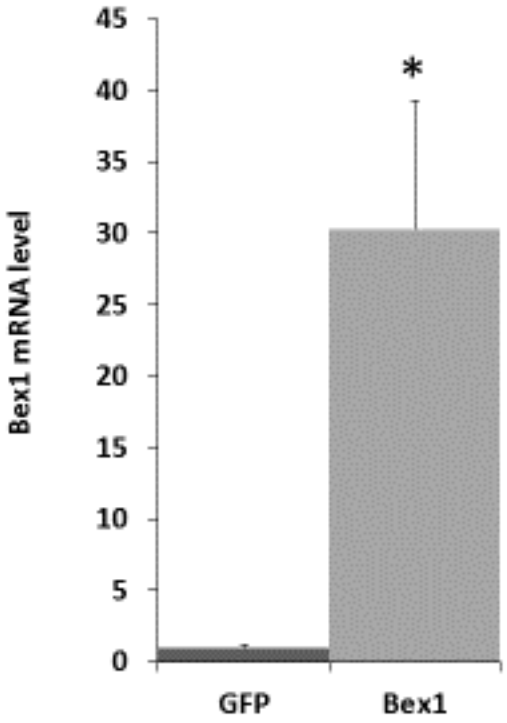

Figure 3.4 A The Expression Level of Bex1 Transcript Was Increased after Infection of Adeno-virus Expressing Bex1.
Primary myoblasts were cultured and infected with virus expressing GFP and Bex1 for 48hs. RNAs were extracted and compared between GFP and Bex1 groups. N=3 independent experiments. Error bars represent s.e.m. *P<0.05.

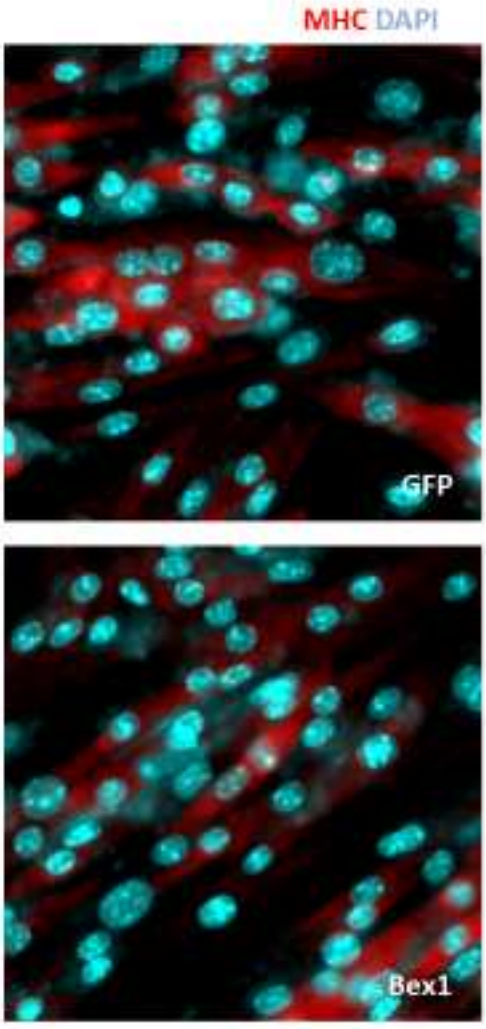

Figure 3.4 B The Bex1 Over-expression Does Not Affect Myoblast-myoblast Fusion.
Primary myoblasts were cultured and differentiated with viruses expressing GFP and Bex1 upon confluence. After 24hs differentiation, cells were immunostained with antibody recognizing MHC (red). Nuclei were counterstained with DAPI (cyan).

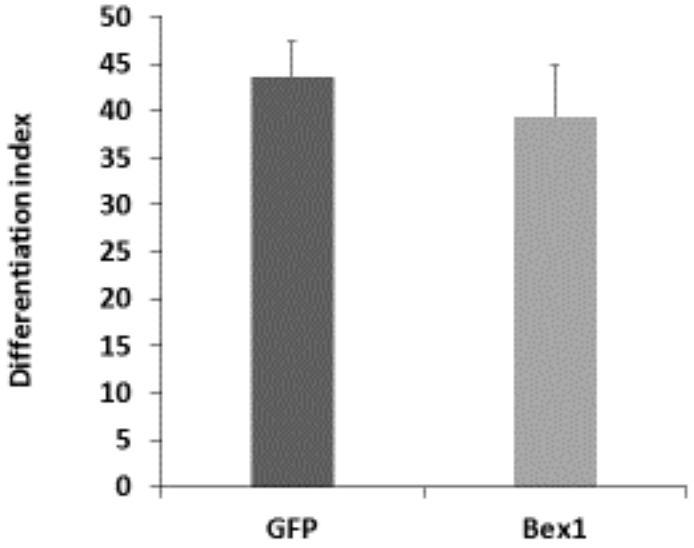

Figure 3.4 C The Bex1 Over-expression in Myoblast Does Not Affect Early Differentiation

Primary myoblasts were cultured and differentiated with viruses expressing GFP and Bex1 upon confluence. After 24hs differentiation, differentiation index was calculated as the ratio of nuclei in Myosin+ cells. n=3 independent experiments.

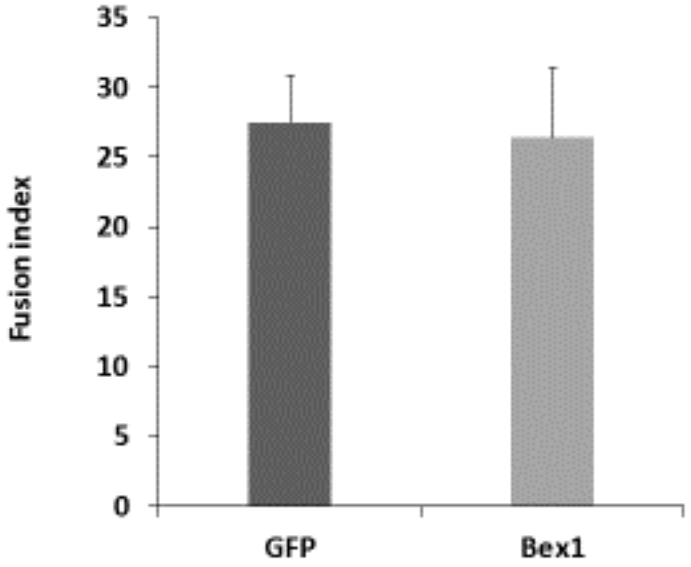

Figure 3.4 D The Bex1 Over-expression in Myoblasts Does Not Affect Primary Fusion.
Primary myoblasts were cultured and differentiated with viruses expressing GFP and Bex1 upon confluence. After 24hs differentiation, fusion index was calculated as the ratio of nuclei in Myosin+ myotubes. n=3.

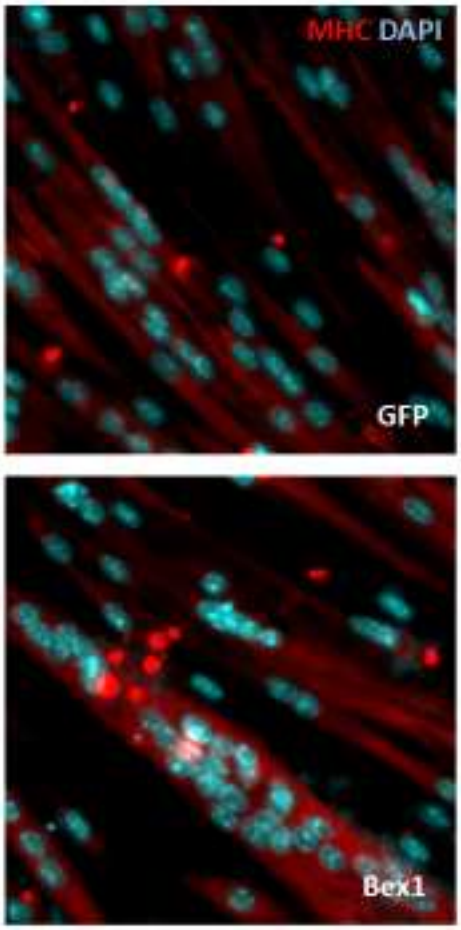

Figure 3.4 E The Bex1 Over-expression Promotes Myoblast-myotube Fusion.
Primary myoblasts were cultured and differentiated upon confluence. After 24hs differentiation, adeno-virus expressing GFP and Bex1 was added with 96 more hours differentiation and then immunostained with the antibody recognizing MHC (red). Nuclei were counterstained with DAPI (cyan).

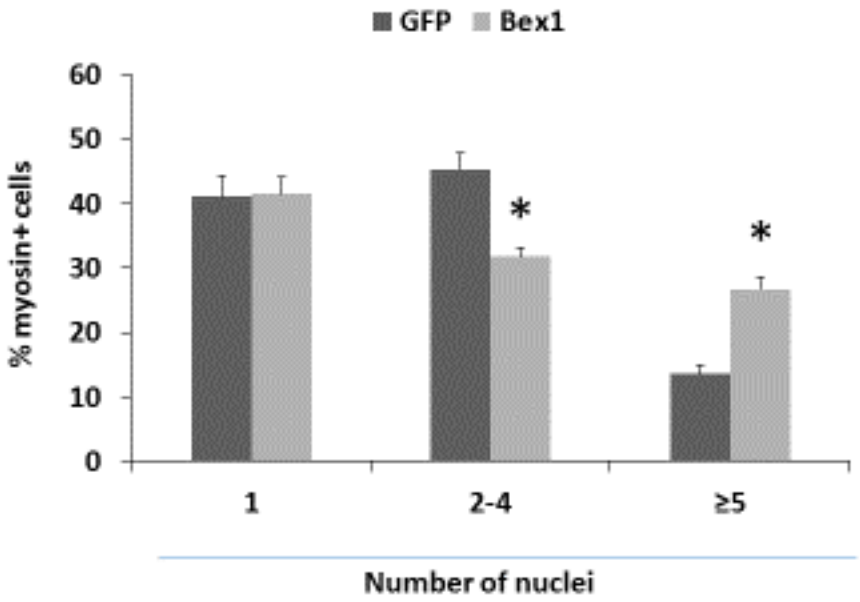

Figure 3.4 F The Bex1 Over-expression Increases Myoblast-myotube Fusion.
Primary myoblasts were cultured and differentiated with infection of virus expressing GFP and Bex1. Percentages of myosin+ cells with different nuclei number in each field were calculated. ≥ 200 cells were analyzed with 5 independent experiments. Error bars represent s.e.m. *P<0.05 compared with GFP-infected cells.

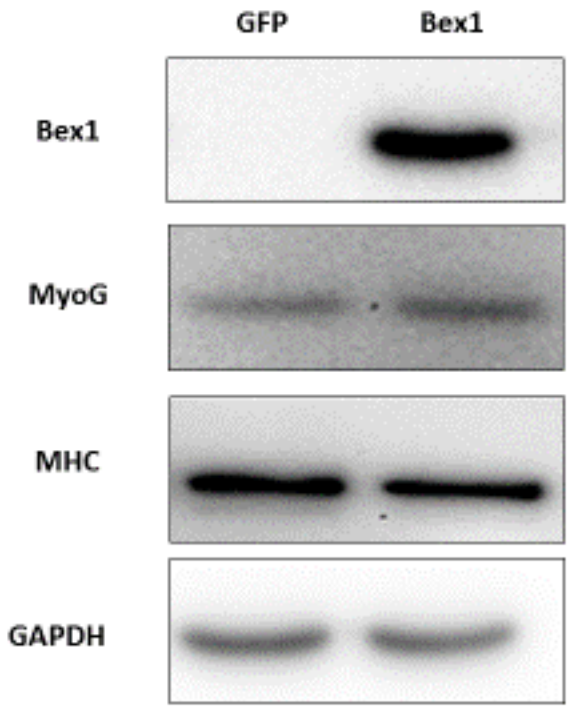

Figure 3.4 G The Bex1 Over-expression Does Not Affect Myoblast Differentiation *per se*.
Primary myoblasts were cultured and differentiated with infection of virus expressing GFP and Bex1. Proteins were extracted after differentiation and subjected to western blot analysis. GAPDH worked as a control. N=3 independent experiments.

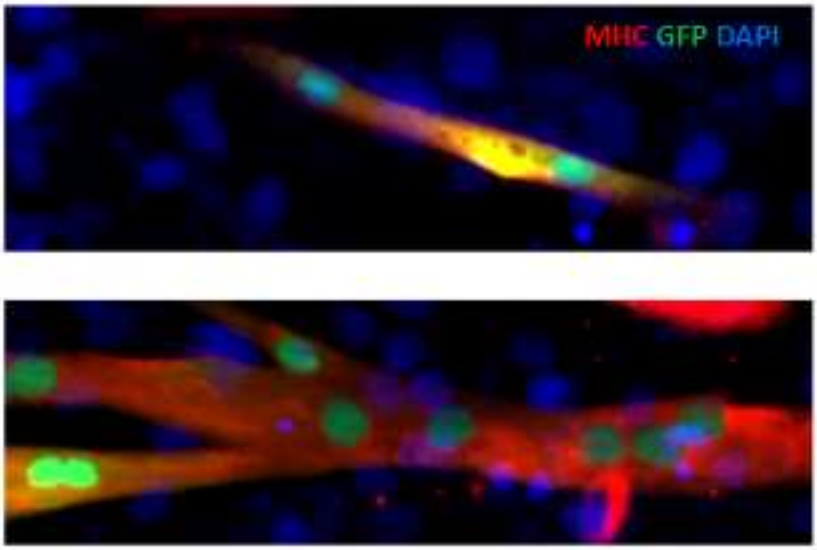

Figure 3.4 H The Bex1 Over-expression Promotes Fusion in C2C12 Myoblasts.
C2C12 myoblasts were electroporated with plasmids expressing GFP and GFP-Bex1 then induced for differentiation for 6 days. Myotubes were immunostained with the antibody recognizing MHC (red). Nuclei were counterstained with DAPI (blue).

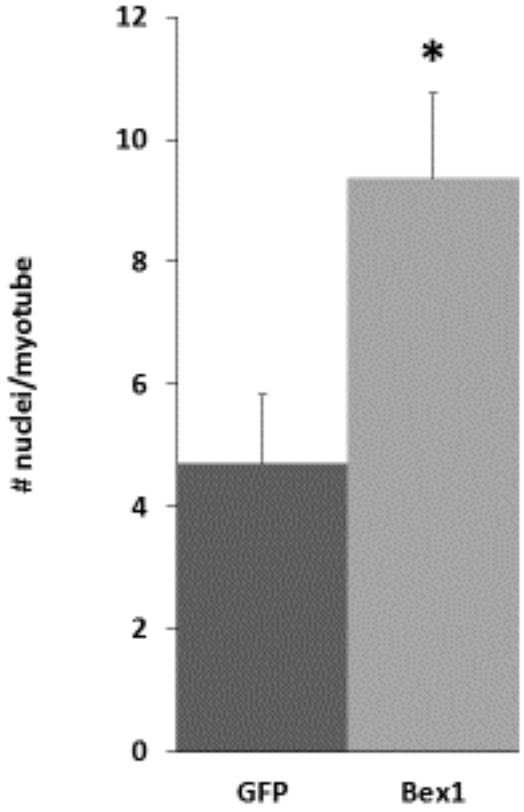

Figure 3.4 I The Bex1 Over-expression Increases the Number of Nuclei in C2C12 Myotubes.
After immunostaining with the MHC antibody, the numbers of nuclei in GFP+ and GFP-Bex1+ myotubes were calculated. N=3 independent experiments. Error bars represent s.e.m. *P<0.05 compared with GFP plasmid transfected cells.

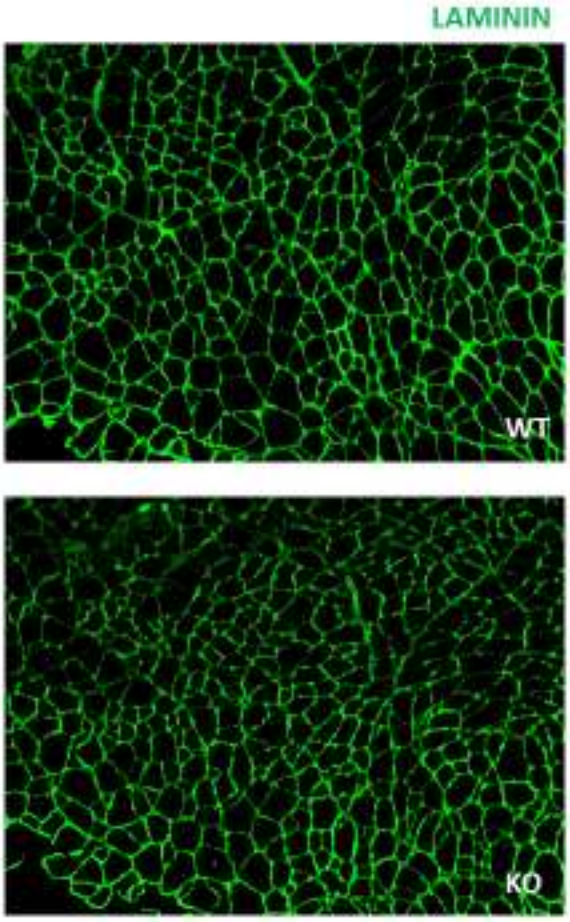

Figure 3.5 A The Myofiber Size in Bex1 Knockout Mice Appears Normal after Regeneration.
TA muscles from WT and Bex1 Knockout mice were injected with CTX. After 14 days regeneration, TA muscles were sectioned and stained with antibody against laminin (green). N=3 mice.

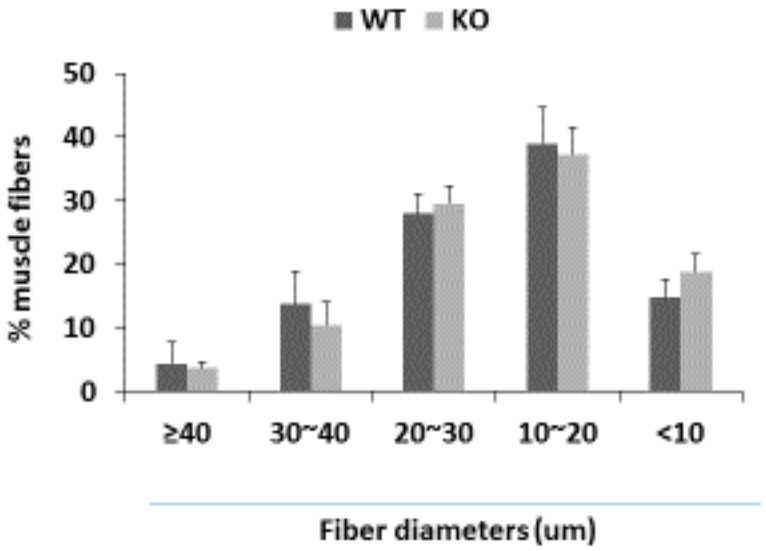

Figure 3.5 B Bex1 Knockout Mice Do Not Exhibit Smaller Myofibers after Regeneration.
After CTX-induced muscle regeneration for 14 days, TA muscles were sectioned and stained with antibody against laminin (green). The fiber sizes were calculated by ImageJ plus software. N=3.

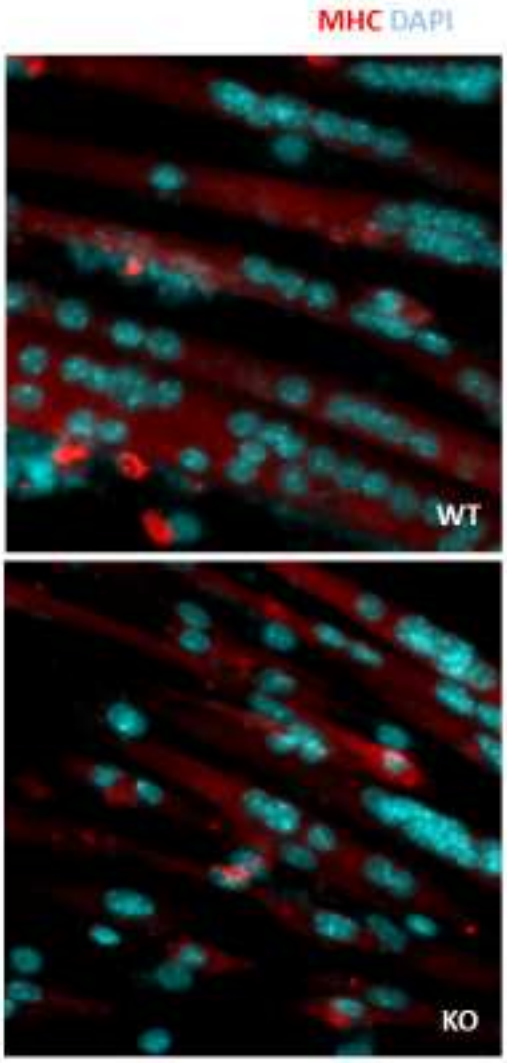

Figure 3.5 C Primay Myoblasts of Bex1 Knockout Mice Are Defective in Myoblast-myotube Fusion.
Primary myoblasts were cultured from WT and Bex1 knockout mice and differentiated for 72hs upon confluence. Myotubes were Immunostained with the antibody recognizing MHC (red). Nuclei were counterstained with DAPI (cyan).

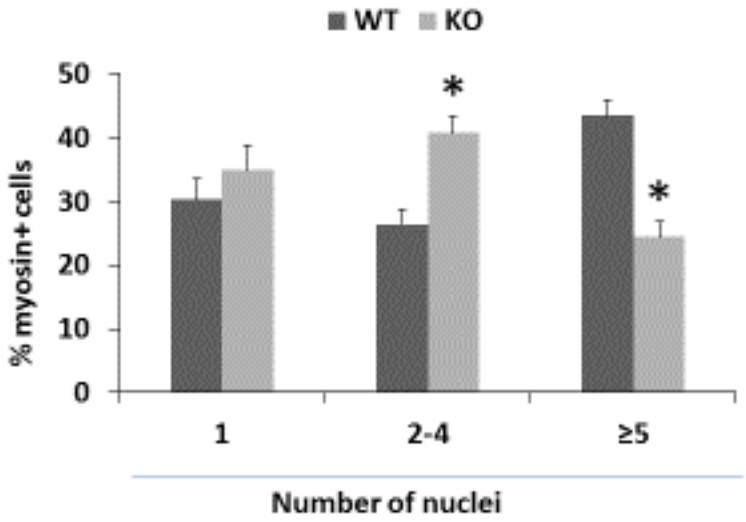

Figure 3.5 D Bex1 Knockout Leads to the Defective Myoblast-myotube Fusion.
Primary myoblast from WT and Bex1 knockout mice were cultured and differentiated for 72hs upon conflunce. Percentages of myosin+ cells with different nuclei number in each field were calculated. ≥ 200 cells were analyzed with 3 independent experiments. Error bars represent s.e.m. *P<0.05 compared with WT cells.

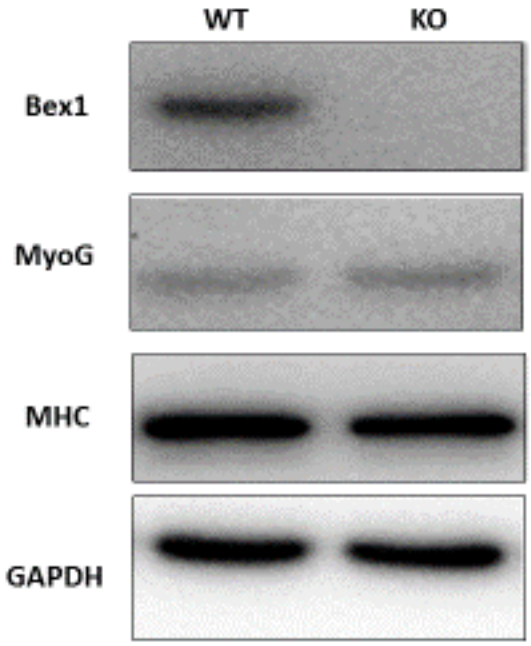

Figure 3.5 E Bex1 Knockout Does Not Affect Myogenic Differentiation *per se.*
Primary myoblasts were cultured from WT and Bex1 Knockout mice, then differentiated upon confluence. Proteins were extracted after differentiation and subjected to western blot analysis. GAPDH worked as a control. N=3 independent experiments.

CHAPTER 4. IMPLICATION AND FUTURE DIRECTIONS

4.1 Implication

Muscle regeneration is a highly orchestrated process involving multiple types of cells, including satellite cells and a poorly determined collection of cell populations. In response to environmental cues, those cells could adapt and cooperate with each other to maintain or recover homeostasis in skeletal muscles, wherein a spectrum of signaling pathways and transcriptional factors have been determined to regulate cellular behaviors. Of note, satellite cells play pivotal roles in contributing to muscle regeneration and satellite cell activation, proliferation and differentiation are precisely controlled by a cellular and molecular regulatory network. However, under some pathophysiological or aging conditions, the delicate balance of the cellular and molecular regulatory network could be perturbed so that muscle wasting may ensue, which is extremely detrimental to health and may even lead to death. The studies in this dissertation aim to explore the cellular and molecular mechanism of muscle regeneration in MDX mouse, a well-characterized model for Duchenne muscular dystrophy, as well as Bex1 knockout mouse, a newly-studied model for investigating the novel roles of Bex1 in skeletal muscles. The findings from this dissertation will not only

facilitate understanding the cellular and molecular regulatory network in muscle development and regeneration but also shed light on providing therapeutic avenues for the prevention and treatment of muscle diseases.

4.2 Future Directions

In this dissertation, Notch signaling pathway and Bex1 were identified with altered expression levels during muscle regeneration. This is supported by the fact that both the Notch signaling pathway and Bex1 were up-regulated in dystrophic muscles (Turk et al., 2005). Furthermore, this dissertation introduced the Notch signaling pathway and Bex1 into the regulatory network during specific phases of myogenesis. In detail, Chapter 2 presented that the Notch signaling pathway promoted self-renewal capacity of satellite cells and inhibited myogenic differentiation whereas Chapter 3 elaborated that Bex1 boosts myoblast fusion with myotubes during myogenic differentiation. Besides, this dissertation did not exclude the possibility that the Notch signaling pathway and Bex1 also play important roles in other periods of myogenesis. Indeed, more intriguing hypotheses pertaining to the Notch signaling pathway and Bex1 in muscle remain unsolved and are worthwhile for future endeavors. This chapter covers some relevant hypotheses based on the aforementioned studies.

4.2.1 Notch in Skeletal Muscles

Notch signaling pathway is evolutionarily conserved and has been previously implicated in cell proliferation and differentiation as well as cell fate determination (Artavanis-Tsakonas et al., 1999). In the myogenesis, it was reported that Notch is involved in multiple signature events from satellite cells to

myoblasts then to myotubes (Conboy and Rando, 2002; Kitzmann et al., 2006). In the satellite cells, Notch is highly active in promoting self-renewal capability through direct regulating Pax7 and inhibiting premature myogenic differentiation partially by targeting MyoD (Wen et al., 2012). Conversely, the mouse with Notch blockage in satellite cells displays dystrophic muscle phenotypes and spoiled muscle regeneration (Lin et al., 2013).

Consistently, Chapter 2 in this dissertation proved that Notch signaling pathway in MDX was disturbed, which led to the self-renewal defects and progressive depletion of satellite cell pool. Constitutive activation of Notch rescued the self-renewal defect but deteriorated muscle regeneration, which was largely attributable to the lasting inhibition of myogenic differentiation (Wen et al., 2012). Nevertheless, this study pointed to a crucial role of Notch in maintaining the pool size of satellite cells for long-term treatment of DMD.

Furthermore, given that muscle regeneration could be improved by the temporarily activation of Notch through the antibody against Notch 1 receptor (Conboy et al., 2003), we interpret that Notch activation within a short time period may be a promising direction when Notch is manipulated to treat muscular dystrophy. To do that, the proteins of Notch ligands can be synthesized. And, the pharmacological activators of Notch can be developed. These reagents will activate Notch temporarily, and facilitate satellite cell self-renewal without progressively blocking myogenic differentiation. Besides, one potential application is to apply these Notch temporal activators to satellite cell

transplantation, which might increase the cell survival rate and contribute muscle regeneration more efficiently.

Another interesting future endeavor can be made to search for new target to balance the self-renewal and myogenic differentiation of satellite cells. As the Wnt signaling pathway is also highly active in muscle regeneration and plays roles in antagonizing the Notch pathway to initiate terminal differentiation. Notch pathway activation combined with Wnt pathway manipulation may be conducive to improve muscle pathology of MDX in terms of not only rescuing self-renewal but also promoting myogenic differentiation. This could be feasible thanks to the bridging roles of GSK3-β between Notch and Wnt pathways as GSK3-β can not only interact with NICD to regulate Notch target genes but also plays pivotal roles in stabilizing β-catenin to activate Wnt signaling pathway (Espinosa et al., 2003; Foltz et al., 2002). Therefore, manipulation of GSK3-β in myogenic lineage cells in a spatiotemporal manner could be promising in the treatment of dystrophic muscle in the both long term and short term runs.

4.2.2 Bex1 in Skeletal Muscles

As a novel gene studied in muscle development and regeneration, Bex1 has a relatively specific functional pattern as the expression of Bex1 is limited to the stage of myogenic differentiation, which is evidenced by the observation of both in *vivo* and *in vitro* in Chapter 3. Chapter 3 also reported that Bex1 has no dramatic regulatory effects on the expression of myogenic differentiation markers, such as myogenin and MHC. Interestingly, Bex1 could positively regulate myoblast fusion with growing myotubes. Myoblast fusion plays fundamental roles

in both muscle development and muscle regeneration. The fusion process requires a series of cellular events including migration, alignment, adhesion and membrane fusion. It is still completely unknown in which specific stages of fusion Bex1 is involved.

Currently, although various signaling pathways and transcriptional factors have been identified to regulate the fusion process, the cellular and molecular mechanism of myoblast fusion is still largely unknown. A good study model of myoblast fusion is Drosophila, wherein researchers have characterized two parallel signaling pathways of Mbc (myoblast city)/Rac and Loner/Rac (Chen et al., 2003; Hakeda-Suzuki et al., 2002; Rushton et al., 1995). Due to the conservation of muscle fusion regulation through species, those two pathways also have significant relevance to study myoblast fusion in mammals. As such, it will be of interest to target the interaction of Bex1 with potential players in these two pathways.

The current cellular and molecular knowledge of Bex1 has elicited us to hypothesize that Bex1 could regulate myoblast fusion through these well-established signaling pathways. For instance, the interaction of Bex1 and calmodulin suggested that Bex1 may be implicated in the calcium signaling pathway (Koo et al., 2007), which has been corroborated to regulate fusion through various targets, including NFATc2 isoform and N-cadherin (Horsley et al., 2003; Mege et al., 1992). Of note, the calcium-dependent N-cadherin could interact with Loner to mediate the fusion process (Dottermusch-Heidel et al., 2012), indicating a possible mechanism of Bex1 function. Thus, an in-depth

investigation of how Bex1 regulates myoblast fusion should be attempted according to the biochemistry and cellular biology knowledge associated with Bex1.

Besides the roles of Bex1 in the fusion characterized in this dissertation, there might be other intriguing hypotheses about Bex1 in the muscle regeneration. For example, Bex1 is abundantly expressed in neurons, which are closely related to muscle development and regeneration. The motor neuron and its innervated muscle fibers comprise the motor unit. The motor neurons play key roles in the muscle function as well as muscle growth, especially the distribution of muscle fiber types (Burke et al., 1971; Calabria et al., 2009; Sohal and Holt, 1980). As Bex1 knockout mice displayed a compromised endurance in the Treadmill-running tolerance test (24% lower than WT) (Koo et al., 2007), it is plausible to hypothesize that the absence of Bex1 might lead to deficient motor neurons, which consequently impairs the muscle performance. These studies might have great implications in searching for therapeutic targets for the treatment of neuromuscular diseases as well as other metabolic diseases.

4.3 Conclusion

This dissertation focused on the roles of the Notch signaling pathway and Bex1 in the muscle regeneration. In this chapter, more intriguing hypotheses have been covered in this chapter and requires further studies to verify. With the increasingly understanding of muscle biology and more advanced technologies in the future, those hypotheses will be tested more precisely and efficiently. Those

results will definitely enrich our scope of knowledge about the cellular and molecular mechanism of muscle development and regeneration.

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Research Experience

- Characterizing satellite cell behaviors in muscular dystrophy.
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- Exploring the regulation of satellite cells by extracellular matrix.

Publications

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