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The role of HIF1alpha and HIF2alpha in muscle development and satellite cell function

Shiqi Yang
Purdue University

THE ROLE OF HIF1α AND HIF2α IN MUSCLE DEVELOPMENT AND SATELLITE CELL FUNCTION

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Shiqi Yang

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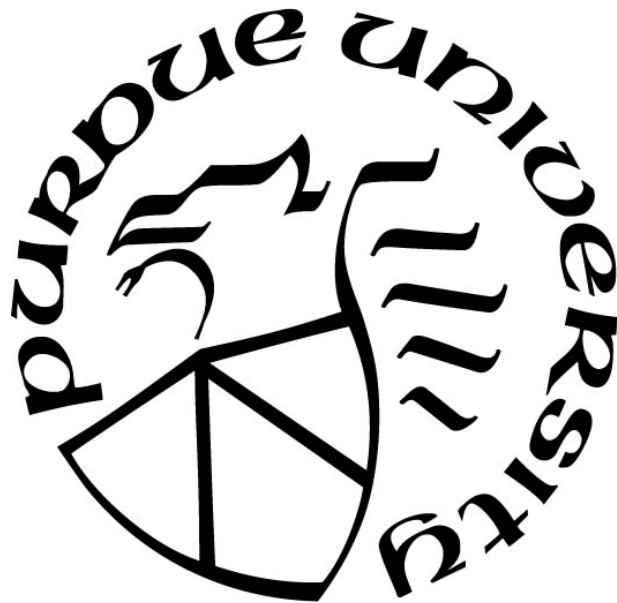
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STATEMENT OF THESIS APPROVAL

Dr. Shihuan Kuang
> Department of Animal Sciences

Dr. Casey M. Theresa
> Department of Animal Sciences

Dr. Kee-Hong Kim
> Department of Food Sciences

Approved by:
> Dr. Ryan Cabot
>> Head of the Departmental Graduate Program

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ABSTRACT

Author: Yang, Shiqi. MS
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Major Professor: Shihuan Kuang

Hypoxia inducible factors (HIFs) are central mediators of cellular responses to fluctuations of oxygen, an environmental regulator of stem cell activity. Muscle satellite cells are myogenic stem cells whose quiescence, activation, self-renewal and differentiation are influenced by microenvironment oxygen levels. However, the in vivo roles of HIFs in quiescent satellite cells and activated satellite cells (myoblasts) are poorly understood. Expression analyses indicate that HIF1α and HIF2α are preferentially expressed in pre- and post-differentiation myoblasts, respectively. Interestingly, double knockout of HIF1α and HIF2α (HIF1α/2α dKO) in embryonic myoblasts results in apparently normal muscle development and growth. However, HIF1α/2α dKO in postnatal satellite cells impairs injury-induced muscle repair, accompanied by a reduced number of myoblasts during regeneration. Analysis of satellite cell dynamics on myofibers confirms that HIF1α/2α dKO myoblasts exhibit reduced self-renewal but more pronounced myogenic differentiation under hypoxia conditions. Mechanistically, HIF1α/2α dKO blocks hypoxia-induced activation of Notch signaling, a key determinant of satellite cell self-renewal. Constitutive activation of Notch signaling can rescue HIF1α/2α dKO induced inhibition of satellite cell self-renewal. Together, HIF1α and HIF2α are dispensable for muscle stem cell function under normoxia, but are required for maintaining satellite cell self-renewal under hypoxic environment

CHAPTER 1.   LITERATURE REVIEW

1.1   Skeletal Muscle: Structure and Function

Skeletal muscle, smooth muscle and cardiac muscle are three main muscle types in vertebrate animals. Skeletal muscle is named so because of its tight connection to the skeleton. As skeletal muscle contracts by stimulation of the nervous system, it pulls or pushes the skeleton to which it is connected, providing energy to move or hold a posture (Kanning et al., 2010). Skeletal muscle can drive skeleton movement due to two main motivational function proteins, actin and myosin (Higashifujime, 1981). Actin is tightly connected long fiber-shaped proteins, as is myosin (Hartman and Spudich, 2012; Howard, 1997). Actin and myosin are arranged side by side with partly overlapping areas, forming one myofibril. Once stimulated by the nervous system, actin pulls myosin from end to center (Hartman and Spudich, 2012). Thus the shape of the muscle will be changed from thin and long to thick and short, which will further change the corresponding location of the skeleton. By this, animals can either hold a posture or make a movement (Hartman and Spudich, 2012).

Mature skeletal muscle cells are fiber-shaped multinuclear cells, which are called myofibers, the largest cells in vertebrate body (Schiaffino and Reggiani, 2011). Each myofiber contains many myofibrils. Myofibrils, as mentioned above, are the functional proteins made of actin, myosin and other proteins that can contract and relax (Howard, 1997). When myofibrils contract, the shape of the myofibers consequently change. Each myofiber has variant numbers of nuclei (Capers, 1960). Each nucleus is supposed to have a functional domain (Hall and Ralston, 1989; Pavlath et al., 1989). Thus it's hypothesized that the size of the myofiber is controlled by the nucleus number (Gundersen and

Bruusgaard, 2008). Each myofiber is surrounded by a basal lamina layer. Between the cell membrane of the myofiber and the basal lamina layer are located the quiescent muscle stem cells called satellite cells (Katz and Miledi, 1961). During skeletal muscle development or regeneration, satellite cells activate, proliferate, differentiate and fuse into myofibers to support the new myofiber formation (Hawke and Garry, 2001). Several myofibers are surrounded and bundled by perimysium forming fascicles. Blood vessels are rich in the gaps between each fascicle. Fascicles are bundled by the epimysium. On each end of the skeletal muscle, the muscle is tightly connected to the skeleton by tendons.

To achieve different functions, different muscles have their own motivational features. So far, four types of myofibers have been determined: type I, type II a, type II b and type II x (Schiaffino and Reggiani, 2011). In terms of the different twitch rates, type I myofibers are usually considered as slow twitch myofibers, while the other three are classified as fast twitch myofibers. Besides the contracting rates, the main metabolic difference between slow myofibers and the fast myofibers is that fast twitch myofibers have a higher level of glycolytic energy metabolism, which shortens the fiber twitch endurance. Slow twitch myofibers exhibit longer endurance due to a more oxidative respiration metabolism (Schiaffino and Reggiani, 2011). Thus, slow twitch myofibers require more oxygen supply, which, consequently, are enriched blood vessels and exhibit a red color. More satellite cells are resident in slow twitch myofibers in the same muscle compared with fast twitch myofibers (Gibson and Schultz, 1982; Snow, 1983). This implies that the difference between mature myofibers may contribute to forming a different microenvironment, which leads to different satellite cell behaviors.

Different muscles contain different ratios of myofiber types. In tibialis anterior muscle, slow twitch possesses a much smaller ratio than in the extensor digitorum longus muscle. This might be due to the different functions of the different muscles. Those muscles that mainly provide powerful force tend to be consisted of fast twitch fibers, while those mainly producing endurance movement muscles tend to contain more slow-twitch fibers.

Unlike myofiber numbers, which are considered unchanging throughout their lifetime, myofiber types can change with age, exercise and health conditions, although the original ratio is set by genotype. During aging, myofiber type tends to switch from slow twitch fibers to fast ones (Payne et al., 2004; Schiaffino and Reggiani, 2011). On the contrary, cycling and continuous exercise can increase slow twitch fiber but decrease fast twitch fibers (Gehlert et al., 2012). Some genomic changes can also switch myofiber types. For example, HIF1α knocking out can switch fast twitch myofibers to the slow ones (Mason et al., 2004; Pisani and Dechesne, 2005). Thus, myofiber types are controlled dynamically.

Besides working as a motivated tissue, skeletal muscle is also a very important thermogenesis tissue. Muscle shivering is one of the two most important heat producing metabolism activities; the other is non-shivering thermogenesis, which is mainly achieved by brown adipose tissue (Astrup et al., 1985; Dubois-Ferriere and Chinet, 1981). Compared with non-shivering heat, muscle shivering is the most important short-term thermogenesis reaction for animals to adapt to short-term dynamic temperature changes. In humans and mice, neonatal babies keep their body temperature mainly by

non-shivering thermogenesis. In other animals, shivering thermogenesis contributes more to keeping body temperature, which mainly relies on muscle (Rowland et al., 2015).

Recently, more studies have revealed that muscle is also a very important endocrine tissue. It has been long known that exercising is good for memory improvement. However, the exact reason is still less known. Only recently, a study group demonstrated that, during exercising, muscle produces more secreted factors called Cathepsin B, which can significantly improve brain cell function and improve memory (Moon et al., 2016). Also, Cathepsin B is helpful for new neuron cell proliferation. Although other reports demonstrated that Cathepsin B is involved in neuron apoptosis, this study provided novel ideas regarding neuro disease therapy applications (Kingham and Pocock, 2001). If this result can be confirmed in humans, we can investigate new methods and applications for brain diseases. All this depends on research into muscle.

Not only for memory improvement, but muscle secretory factors also participate in brown adipose tissue stimulation and beige cell recruitment. FGF21 and FNDC5 are the two of the most important factors involved in this regulation (Fisher et al., 2012; Hanssen et al., 2015; Hofmann et al., 2014). Additionally, suckling can induce the release of FGF21 from the liver in the neonatal stage (Hondares et al., 2010). FGF21 can also be released from muscle, which can improve metabolism by stimulating brown adipose tissue function and recruiting beige cells in white adipose tissue (Hanssen et al., 2015). While FNDC5 is released from muscle when exercising, targeting white adipose tissue induces browning, which can also improve metabolism (Hofmann et al., 2014).

Muscle is a complex organ with complex functions. The health condition of muscle is very important, not only in the muscle itself but also as a whole body regulation

system. Thus, muscle research is necessary and important for human health. With a better understanding of muscle, we can achieve a better human life.

1.2   Skeletal Muscle Stem Cells: Satellite Cell

Skeletal muscle is a complex tissue consisting of multi types of cells. Different cell types conduct different functions, such as myofiber for contraction and twitch, epithelial cells for bundling of the myofiber clusters and endothelial cells for forming blood vessels. Multiple types of stem cells are resident in the muscle, such as fibro-adipogenic progenitors, epithelial stem cells, endothelial stem cells and, the most important in muscle stem cells, satellite cells (Pannerec et al., 2013).

Satellite cells are progenitors to mature skeletal muscle cells. In 1961, skeletal muscle satellite cell was first named based on its location (Katz and Miledi, 1961). These cells were described as satellites, surrounding the myofibers, and were located between the myofiber cell membrane and basal lamina membranes. The first two groups found satellite cells individually in frog and rat limbs using electron microscopes (Katz and Miledi, 1961; Mauro, 1961). Afterwards, different groups have identified satellite cells in other vertebrate animal models (Grounds and Yablonka-Reuveni, 1993).

Upon discovery, people failed to recognize the function of satellite cells due to their commonly quiescent state (Schultz et al., 1978). However, the studies on satellite cells developed quickly afterwards. The satellite cells were identified as myogenic stem cells, given their ability to self-renew and produce mature myofibers (Collins et al., 2005; Grounds and Yablonka-Reuveni, 1993; Yablonka-Reuveni and Rivera, 1994). More studies for satellite cell marker identification and regulation achieved impressive results, consequently. Discovery of specific expressed molecular markers, in particular Pax7 and

Pax3, has facilitated classification and isolation of satellite cells using flow cytometric sorting (Kassar-Duchossoy et al., 2005; Seale et al., 2000). Moreover, the function of different molecular pathways involved in satellite cell regulation has been clarified, including Notch, Wnt and TGF β(Allen and Boxhorn, 1987; Conboy and Rando, 2002a; Otto et al., 2008b; Rathbone et al., 2011).

More recently, satellite cells have been shown as multipotent stem cells, which can not only differentiate into mature myocytes, but also can undergo adipogenesis induced by an adipogenic induction medium and osteogenesis cultured in an osteogenic induction medium in vitro (Asakura et al., 2001). However, the multi-potency of satellite cells in animals has never been illustrated, even though this finding provides a new idea to broaden the application of satellite cells in transplantation therapies of different tissues. This finding also provides knowledge of how satellite cells work and the regulation of satellite cells' self-renewal and differentiation.

1.2.1 Development of original of satellite cells

Embryonic muscle development can be separated into two main stages. During the first stage, the primitive muscles are formed. The primitive muscle cells come from the dermomyotome and are located underneath the dermomyotome region (Gros et al., 2004). Some cells in the outer layer of the dermomyotome, expressing Myf5, migrate out and form the initial muscle. The primitive muscles then become the primary myotome and are the original skeletal muscles in trunk (Gros et al., 2004). In the second stage of muscle growth, epithelial-to-mesenchymal transition (EMT) occurs in the central part of the dermomyotome (Gros et al., 2005). During this process, tightly connected epithelial cells separate, moving and developing into muscles in different body region (Ben-Yair

and Kalcheim, 2005; Kalcheim and Ben-Yair, 2005). The trunk skeletal muscle originates

from the cells in the central part of the dermomyotome, and the rest cell population of the

dermomyotome may then be determined to differentiate into brown fat or dermis (Wang

et al., 2014a). While the limb skeletal muscle descends from the cells in the lateral part of

dermomyotome, other cells in this part will develop into endothelia muscles (Ben-Yair

and Kalcheim, 2005; Esner et al., 2006; Gros et al., 2005). However, the regulation of the

cell fate decision and the control of migration of these cells is still unknown. One

hypothesis is that the asymmetric division may contribute to the migration and cell fate

decision (Cinnamon et al., 2006).

Cells originally migrating from the central dermomyotome are resident in the

primitive muscles. These cells express Pax7 and Pax3, working as the original committed

skeletal muscle stem cells (Relaix et al., 2005). Some of these stem cells proliferate

continuously, leading to an increased stem cell population and contributing to the early

fetal stage muscle development. During this kind of proliferation, some cells lose their

stemness and differentiate into myocytes while other cells keep their stem cell character.

These cells are resident underneath the basal lamina and they are widely considered as

the original adult muscle stem cells. The specific markers of these cells are Pax3 and

Pax7 (Relaix et al., 2005).

Although both Pax3 and Pax7 positive cells can differentiate into mature muscle

cells, there is a difference between these two groups. Scientist have found that Pax3

positive cells can not only differentiate into myocytes, but also they have the potency to

develop into vascular cells and other cell types (Hutcheson et al., 2009; Relaix et al.,

2004; Relaix et al., 2005; Schienda et al., 2006). This implies that Pax3 positive cells

obtain more multi-potency than Pax7 positive cells. This theory has been supported by the fact that, after E11.5 in the development stage, some Pax3 Pax7 double positive cells turn into Pax7 single positive cells and are committed to undergo the muscle development process (Relaix et al., 2004).

Although it has been widely accepted that Pax7 positive cells are the main source of satellite cells, it is possible that some other cells can contribute to muscle formation. Some other cell types can be induced to myocytes robustly under cell culture conditions (Esner et al., 2006). Thus, it is still too early to make a conclusion that all muscles come from Pax7 lineage cells.

During fetal development and the neonatal development stage, some composition of Pax7 positive muscle stem cells differentiate into myocytes. When Pax7 positive cells express Myod, they withdraw from the cell cycle to differentiate into myocytes and lose their ability to proliferate (Sabourin and Rudnicki, 2000). Thus, during muscle development, the population of Pax7 positive is reduced. There are still certain portions of muscle stem cells that can maintain their proliferating ability, however, even though they stay quiescent in their normal condition.

To isolate muscle stem cells in adults, besides Pax7 there are other cell surface markers that can be used. Even different markers can be used to identify different subpopulation linages of satellite cells. The common surface markers for satellite cells are CD34 and integrin alpha 7 (Beauchamp et al., 2000; Sacco et al., 2008). Double positive cells exhibit muscle stem cell features and can be induced into myocytes in culture conditions. Early studies have demonstrated that satellite cells in adult muscle are heterogeneous, and some of the satellite cells are more "stem" than others, exhibiting a

higher transplant efficiency (Collins et al., 2005). The satellite cells never express Myf5, exhibiting more stemness compared to Myf5 linage satellite cells, which contributes to the satellite cells' population maintenance during regeneration. The lower stemness satellite cells may be responsible for the growth and repair of myofibers under normal conditions (Kuang and Rudnicki, 2008; Kuang et al., 2007).

There are two trends in satellite cell therapy applications. One is to modify satellite cells to increase satellite cell function. The other is to transplant high performance satellite cells to increase efficiency. Both of these two trends need a deeper understanding of satellite cells regulation and origination.

1.2.2 Satellite cells function and metabolism

Satellite cells, as mentioned before, are the main source of myocytes and myofibers during development and regeneration. During development, satellite cells differentiate into mature myocytes and fuse into myofibers continuously triggered by different pathways (Sabourin and Rudnicki, 2000). The undifferentiated satellite cells proliferate and self-renew to maintain the satellite cell pool.

In mature skeletal muscle, satellite cells resident between basal lamina and myofiber normally keep a quiescent state. Upon injury, the quiescent satellite cells are activated and proliferate. Proliferating satellite cells undergo either differentiation to fuse into myofibers to repair destroyed myofibers or stay undifferentiated and maintain satellite cell population to keep homeostasis and prepare for future need (Charge and Rudnicki, 2004).

During the development and homeostasis stage, skeletal muscle stem cells undergo either differentiation for new myofiber formation or self-renewal for stem cell

pool maintenance. These two metabolic process are controlled by two different but important groups of factors. Factors that can initiate, promote and maintain myoblast myogenic differentiation are called muscle-specific regulatory factors (MRFs) (Pownall et al., 2002). Transcription and activation of MRFs is controlled as a cascade. The first activated gene is Myod, followed by activation of Myog and Myogenin (Berkes and Tapscott, 2005; Nabeshima et al., 1993). The activation of Myod determines satellite cell fate to undergo myogenic differentiation. Knocking out Myod can abolish the myogenic differentiation capacity of myoblasts. The blocked myogenic differentiation can be retrieved by ectopic Myod expression (Davis et al., 1987; Tapscott et al., 1988). It has been reported that Myod-deficient myoblasts can be induced to adipocytes when cultured in adipogenic induction media. However, whether Myod deficiency is pushing myoblasts to the adipocyte side or dedifferentiates myoblasts to multipotent stem cells is still unknown. Besides Myod, Myf5 is also considered another important regulator. Abolishing Myf5 can induce abnormal muscle development (Braun et al., 1992; Kablar et al., 1998; Rudnicki et al., 1993). The function of Myod and Myf5 is overlapped insomuch that single knocking out mice can still survive despite abnormal development. However, their common downstream target gene, Myogenin, is critical in muscle development, such that Myogenin deficiency induces fetal death due to severe inhibited muscle development (Hasty et al., 1993; Nabeshima et al., 1993). Different than the MRFs, Pax7 keeps satellite cells in an undifferentiated stage and keeps the ability to proliferate and self-renew (Olguin and Olwin, 2004). Pax7-deficient mice exhibit impaired muscle development and muscle regeneration (Kuang et al., 2006). Also, in physiological conditions, proliferating or quiescent satellite cells keep expressing Pax7 (Zammit et al.,

2006), while the shade of Pax7 is a marker that satellite cells withdraw from to cycle and undergo myogenic differentiation (Zammit et al., 2006).

1.2.3 Satellite cells dysfunction and disease

Satellite cells are the source and engine of skeletal muscle. Accurate function of satellite cells is very important for skeletal muscle homeostasis. Satellite cell dysfunction definitely leads to severe muscle disease. The dysfunction of satellite cells may come from a genetic mutant in the genome or may be induced by abnormal microenvironment changes. In terms of the impaired function of satellite cells due to the microenvironment, the types of growth factors involved include Wnts, FGFs and TGF-β factors (Amthor et al., 2013; Brack et al., 2007; Liu et al., 2001; Toth et al., 2011). These factors cooperate with each other to maintain accurate muscle functions. Lack of or an exceeded level of any of these factors may lead to muscle changes and dysfunction. For example, deficiency of myostatin can cause muscle hypertrophy and can exhibit the double muscle phenotype (Amthor et al., 2013). FGF2 can largely promote satellite cell proliferation, and a lack of FGF2 can lead to less efficient muscle regeneration (Yablonka-Reuveni et al., 2015). During aging, the Wnts level is largely elevated, which leads to a lower satellite cell self-renewal ratio (Brack et al., 2007). Besides the circulation growth factors, oxygen levels also effect satellite cell function. It has been determined that low oxygen levels could largely inhibit myogenesis, and thus animals in high altitudes may have less efficient muscle regeneration than those in low altitude levels (Di Carlo et al., 2004). Also, in vitro studies have shown that hypoxia or low oxygen levels can increase satellite cells' self-renewal, which may be helpful to keep the satellite cell pool and further keep muscle younger (Liu et al., 2012b).

During aging, skeletal muscle stem cells gradually lose their self-renewal capacity. The satellite cell pool dramatically decreases as animals get older. Different mechanisms involved the loss of satellite cells have been determined by different groups. The regulation mechanisms of Notch and Wnt signaling have been widely accepted. During aging, satellite cells exhibit lower Notch levels but more active Wnt signaling. Both Notch activators and Wnt inhibitors can partly rescue the aging-induced loss of satellite cell stemness (Brack et al., 2007).

Efficient skeletal muscle function is a prerequisite for a healthy life. However, many skeletal muscle-related diseases affect human health, such as Parkinson's disease, muscle ischemic diseases and muscle weakness. Many of these diseases are originally caused by sickness in other tissue, and subsequently impair muscle function. For example, the originality of Parkinson's disease is central neuron system dysfunction. But the abnormal nervous system could further cause the shaking of the muscle. Long-term denervation can induce muscle atrophy and dysfunction (Schulz-Schaeffer, 2016).

Among these diseases that exhibit phenotypes in skeletal muscle, Duchenne muscular dystrophy or Duchenne syndrome (DMD) is a disease that exactly originates from muscle dysfunction due to satellite cell dysfunction (Ciafaloni et al., 2016). DMD is an inherited disease caused by a mutation in the dystrophin protein. Dystrophin connects inner cellular motivational proteins with the extracellular matrix. Dystrophin deficiency can induce continuous satellite cell differentiation, which deplete satellite cell pools and induce muscle weakness and death (Blake et al., 2002).

Besides DMD, there are other similar but less severe muscle diseases, such as Becker muscular dystrophy, congenital muscular dystrophy, myotonic muscular

dystrophy (MMD), distal muscular dystrophy and so on (Nowak and Davies, 2004; Schulz-Schaeffer, 2016). Some of these diseases are also caused by the mutation of dystrophin, such as Becker muscular dystrophy (BMD). The difference between BMD and DMD is that the mutation of dystrophin in BMD can only impair skeletal muscle function in old age and only happened in males (Nowak and Davies, 2004). Besides BMD, others may be caused by different gene mutations. For example, MMD, the most common adult muscle dystrophy, is caused by the DNA mutation of DMPK (type I MMD) or ZNF9 (type II MMD) (Turner and Hilton-Jones, 2010). With distal muscular dystrophy, patients have genome mutations that may cause the disconnection of the dystrophin-glycoprotein complex, further inducing muscle dysfunction.

So far, there are no efficient valid therapy applications that can clinically cure DMD. However, researchers have proposed several potential methods to treat DMD. Especially since the discovery and applications of iPS cells, researchers have hoped to manipulate and refine the stem cells isolated from patients and inject them back to replace the mutant stem cells in order to rescue skeletal muscle dystrophy.

1.3 Satellite Cell Niche

1.3.1 Satellite Cells Microenvironment

Satellite cells are located between the basal lamina and the myofiber membrane, tightly surrounded by these two membranes. These two membranes encapsulate the satellite cells, forming a microenvironment that regulates satellite cell homeostasis, cells' quiescence and activation, as well as self-renewal or myogenic differentiation after activation.

The four main mechanisms involved in this regulation are: attaching scaffold, extracellular matrix, microenvironment growth factors and nutrition. The microenvironment provides an ideal attaching scaffold for satellite cells. This scaffold fuctions to keep satellite cells quiescent. Breaking or injuring the scaffolding may lead to activation and myogenic differentiation of satellite cells. Dystrophin is an important protein involved in the connection of myofibers with the basal lamina. Dystrophin deficiency leads to myofiber damage. If the impaired myofiber fails to keep satellite cells quiescent, it leads to continuous activation and differentiation of satellite cells. Satellite cell depletion leads to failure of muscle regeneration and homeostasis, as well as severe muscle disease.

In addition to the structure of the basal lamina, some proteins are inserted into the basal lamina and the existing muscle fiber and may regulate satellite cells directly. Recently, researchers found that Collagen VI is required for satellite cells' self-renewal and population maintenance (Urciuolo et al., 2013). After knocking out Collagen VI, the mice exhibited impaired muscle regeneration caused by fewer satellite cells self-renewing (Urciuolo et al., 2013). Also, satellite cells cultured in Collagen VI-deficient muscle fiber exhibited less self-renewal. Further, myoblast culture in a null Collagen VI-coated dish showed less self-renewal than in Collagen VI-coated dish. These results indicate that Collagen VI is helpful for satellite cells maintenance (Urciuolo et al., 2013). Even though the reason and how Collagen VI can facilitate satellite cell self-renewal is unknown, these results imply a complex regulation of the microenvironment or ECM involved in satellite cell regulation.

Besides ECM, some important growth factor ligands exist in cell membranes also involved in satellite cell regulation. It has been widely accepted that Notch signaling is very important in satellite cell quiescence and self-renewal. But the exact mechanism of how Notch regulates satellite cells in vivo is still unknown. One hypothesis is that Notch ligands exist in the muscle fibers and are enriched in the satellite cells' resident region (Kuang et al., 2008). Thus, the Notch signaling in satellite cells were continuously activated under normal conditions. This hypothesis can explain why asymmetric division can lead to different cell fates: satellite cells residing nearby fibers are still Notch activated, while the satellite cells detached from muscle fibers will undergo myogenic differentiation due to Notch removal. For symmetric division, both daughter cells attach to muscle fibers, leading to activation of Notch signaling in both cells. Thus, both daughter cells can keep their stem cell capacity (Kuang et al., 2008).

Growth factors of which transport through blood all over the whole body called endocrine, or they can act just nearby, called paracrine. The functional region of endocrine and paracrine is different. However, in microenvironments, both endocrine and paracrine are controlled in terms of concentration, even though the concentration is impacted at the whole body level. Growth factors are highly involved in satellite cell regulation. Fibroblast growth factors (FGFs), WNTs and bone morphogenetic proteins (BMPs) are the most important growth factors in regulating satellite cell function, which I will talk more about in following chapters. These factors can be secreted by the surrounded cells inside the muscle, or they can be transferred from other tissue. The balance of these factors keeps satellite cell homeostasis. During muscle injury, other cells

invade into the muscle and the regulation of the growth factor balance changes. Satellite cells are activated and differentiated to repair injured fibers.

As for nutrition, including amino acids, glucose and oxygen, satellite cell behavior can be changed when challenged by different nutrition levels. Our in vitro experiment demonstrated that during starvation, satellite cells tend to stay quiescent. This result is hard to do in animals, because it's hard to achieve muscle-specific nutrition restriction.

Previous results have shown that under a hypoxia state, myoblast differentiation is largely blocked. The hypoxia inhibited myogenic differentiation is induced by different mechanisms, including accelerated Myod protein degradation, decreased Myod mRNA levels, inhibition of Wnt signaling and elevation of Notch signaling (Di Carlo et al., 2004; Majmundar et al., 2012; Wang et al., 2015). Also, our previous result demonstrated that during hypoxia, satellite cells tended to undergo self-renewal rather than differentiation (Liu et al., 2012b). Hypoxia-promoted self-renewal originates from the promotion of Notch signaling. In vivo studies show consistent results that muscle regeneration in high altitudes is largely impeded due to low oxygen levels (Blaisdell, 2002). Hypoxia-treated satellite cells exhibit higher transplantation efficiency. Both results indicate that oxygen level is very important in satellite cell regulation. Recently, researchers found that NAD+ level can alter satellite cell behaviors (Zhang et al., 2016). The balance of NAD+/NADH is controlled by energy and oxygen levels. NAD+ increased when energy level was low or oxygen level was high. Recent research also has demonstrated that NAD+ repletion can largely improve mitochondrial function and improve muscle stem cell stemness. This group fed mice with NAD+ precursor NR

to increase the NAD+ level of the whole body. Surprisingly, they found that NR supplements can prohibit muscle degeneration. Also, they found that NR delays senescence of neural SCs and melanocyte SCs and increases mouse life span (Zhang et al., 2016).

All these results demonstrate that satellite cell function and behavior regulation is not just programmed in the satellite cell itself, but is largely dependent on the microenvironment changes. Meanwhile, satellite cells can monitor muscle condition by receiving messages from the microenvironment to determine subsequent reactions. Once a muscle is challenged by injury, growth factors, scaffold, cell surface ligands, nutrition and oxygen level, all these factors will change. Satellite cells receive signals from all these changes and then determine the cell fate for muscle repair and, at the same time, maintain the stem cell population for long-term homeostasis. Thus, a deeper understanding of how the microenvironment controls satellite cell cell-fate is required for acute application therapies in muscle injury and muscle disease treatment.

1.3.2   Multipotent Stem Cells Resident in Skeletal Muscle

Skeletal muscle is a complex organ that consists of multiple types of cells. Besides the major muscle-specific progenitor or precursor cells, such as myoblast and satellite cells, multipotent stem cells are also resident in skeletal muscle. These multipotent stem cells were only recently identified, and their function and origin is not clear. However, they are very important in maintaining skeletal muscle homeostasis. Their population needs to be tightly regulated within a certain amount, as too much or too few will lead to muscle dysfunction and impaired muscle regeneration. Although researchers have found some functions of these cells, the exact function and regulation is

still unknown. Even less whether these cells belong to one cell type or are just a mixture of different cell types is unknown. In order to gain a deeper understanding of these kinds of cells, more research needs to be done to investigate the specific function and markers of skeletal muscle resident multipotent stem cells.

The first time researchers found skeletal muscle resident multipotent stem cells was in 2010 (Joe et al., 2010; Uezumi et al., 2010). Two groups found these cell types independently at the same time and they published them in Nature cell biology in a same series. Before that, it was long known that during aging, exotic adipose tissue accumulated in muscle, which can induce insulin resistance and muscle dysfunction, though the origin of the intramuscular fat was unknown. Some groups claimed that the intramuscular fat originated from muscle satellite cells (Asakura et al., 2001). They provided evidence that satellite cells are actually multipotent stem cells. Some other groups believed that the intramuscular fatwas coming from blood (Joe et al., 2010). Until 2010, people found that mesenchymal stem cells resident in skeletal muscle can be induced into adipocytes. Kunihiro Tsuchida's group isolated stem cells using satellite cell marker negative and endothelial marker negative (lin-, CD31-, CD34+, Integrin 7-) as a sorting requirement (Joe et al., 2010). They found that these mesenchymal stem cells can be induced into different cell types, include adipocytes. Their transplantation results demonstrated that mesenchymal stem cells can be induced into adipocytes in muscle by glycerol injection-induced injury in vivo, indicating it was the origin of ectopic muscle fat. Further, they found these cell specifically express PDGFRα, which can be used as a specific marker for isolation (Joe et al., 2010).

Another group got a similar result at the same time. They found that PDGFRα positive cells can be differentiated into both fibroblasts and adipocytes, so they named them fibro/adipogenic progenitors (FAPs) (Uezumi et al., 2010). They found that during muscle regeneration FAPs first increased during the first three days and then decreased to non-injury levels as muscle regeneration finished. The increase of FAPs was very fast and dramatic. The proliferation of FAPs started earlier than satellite cell proliferation, implying that they may be helpful for satellite cell proliferation. Under normal conditions, FAPs proliferate slowly and it only occurs in a few cells. After injury, eighty percent of FAPs started to proliferate, indicating cell state changes. All these changes imply FAPs may react to facilitate muscle regeneration. The researchers didn't mention more, except for an increased level of Wnt and Il-6 in FAPs after injury. Wnts and Il-6 are helpful for satellite cell proliferation. Thus, they concluded that FAPs promote muscle regeneration (Uezumi et al., 2010).

FAPs are adipocyte progenitors. FABP4 (fatty acid binding protein 4, aP2) is a marker of pre-adipocyte and adipocyte. Our group, using aP2Cre to trigger iDTR expression to ablate adipocyte progenitors in skeletal muscle (Liu et al., 2012a). Our results demonstrated that after deletion of aP2 positive cells, muscle regeneration was largely impaired. These results indicated that the adipocyte progenitors in muscle actually contribute to muscle regeneration, although the exact regulation is unknown. Also, why these cells proliferate to that extent after injury is still unknown.

Recently, another group showed the exact trigger that can induce proliferation of FAPs after injury (Heredia et al., 2013). Instead of considering these factors as causing proliferation in the muscle itself, this group connected muscle injury with the immune

system and type II innate immune reaction. They demonstrated that knocking out Il-4 and Il-13 in monocytes can largely decrease the proliferation of FAPs. This kind of decrease can be rescued by an Il-4 and Il-13 supplement. Meanwhile, when there is a lack of Il-4 and Il-13, muscle regeneration is worse compared with the control. Since Il-4 and Il-13 can't affect satellite cells directly, the impaired muscle regeneration should be induced by the lack of FAPs. In the end, they demonstrated that one critical function of FAPs is to engulf debris of dead fibers and work as immune cells. Thus, FAPs can help muscle regeneration by removing damaged fiber debris.

These results demonstrated that enough FAPs are needed for efficient muscle regeneration; however, there are other studies that confirmed that too many FAPs may lead to fibrosis and impeded muscle regeneration or may lead to ectopic fat accumulation in muscle.

Since FAPs are the original of two important diseases, injury-induced fibrosis and ectopic fat accumulation, researchers have begun to look deeper into it. What is the regulation that controls the increase of FAPs when needed, and what is the mechanism that cascades the decrease when FAPs are no longer needed? Very recently, one group found that the increase and decrease of FAPs is controlled by time case and dose case (Lemos et al., 2015). The most important regulator involved in this regulation is TGF-β. When muscle is challenged by injury, TGF-β level increased to induce an increase of FAPs. However, as regeneration goes on, TGF-β level is reduced, inducing apoptosis and a decrease of FAPs. If that's the case, then the muscle exhibits normal regeneration. However, when muscle is challenged by continuous or multiple injury, then the TGF-β will stay high, which may induce fibrosis. This model can explain much of the injury-

induced fibrosis in different tissues. Also, it provides an idea on how to heal injury-induced fibrosis using TGF-β inhibitors to reduce excessive fibroblasts (Lemos et al., 2015).

However, one thing that still can't be explained by this TGF-β induced fibrosis model is that TGF-β can promote fibroblast formation, but how can accumulated FAPs be induced into adipocytes? FAPs are multipotent stem cells, but they can only differentiate into one single cell type under certain conditions. So, the factors and regulation involved in adipogenic induction of FAPs is still largely unknown.

Given the fact that FAPs can be functional in different ways, are these cells truly just one unique cell type or are they actually heterogeneous and we just can't distinguish them from each other? Another very important question is since these cells specifically express PDGFRα, what is the unique function of the PDGF signaling pathway's participation in the regulation of FAPs? With these questions, we believe that as we study more about FAPs, we will be able to treat FAPs-related diseases and push them to a beneficial side rather than the other.

1.4   Signal Pathways Involved in Satellite Cell Regulations.

1.4.1   Notch Signaling Promotes Satellite Cell Self-Renewal

Satellite cell self-renewal is very important in keeping the stem cell population. Different factors participate in the regulation of self-renewal. Among these factors, Notch signaling is one of the most well-studied and clearer understood pathways. However, there are still many myths hiding in these pathways, including crosstalk with other signaling or application methods, which need to be discovered.

Generally, Notch signaling is an evolutionarily conserved mechanism that can be found in almost every animal (Chitnis and Bally-Cuif, 2016; Yuan et al., 2015). This signaling pathway can regulate cell metabolism and subsequent changes by cell-to-cell interactions. So far, the majority of research on Notch signaling are about its cell fate determination mechanism. Only recently, scientists found that Notch signaling actually can regulate energy metabolism by inhibiting thermogenesis (Bi et al., 2014). Thus, Notch signaling may potentially participate in other kinds of cell metabolism. The Notch pathway was first demonstrated in Drosophila melanogaster (Andersson et al., 2011). The Notch protein found in Drosophila is a 300 kDa transmembrane receptor. It contains two main parts: the extracellular domain (NECD) and the intracellular domain (NICD). The extracellular domain, which can recognize extracellular Notch ligands, contains 36 tandem epidermal growth factor (EGF)–like repeats and three cysteine-rich Notch/LIN-12 repeats (Gordon et al., 2008).

The NICD parts consist of six tandem ankyrin repeats, which is shown to be responsible for the cleavage of NICD, one glutamine-rich domain, which is related to binding of cofactors, and a PEST sequence (Gordon et al., 2008). When the NECD part binds to the Notch target ligands, the NICD part will be released and function as a transcriptional regulator binding with other cofactors. The cleavage of NICD is controlled by the activation of γ-secretase. The activation of γ-secretase is mainly controlled by the binding of the Notch receptor and Notch ligands. Notch inhibitors are designed to inhibit the activation of γ-secretase, such as DBZ and DAPT (Yuan et al., 2015), while some intracellular proteins, such as HIF1α, can bind to γ-secretase and activate its cleavage

activity (Villa et al., 2014b). Also, HIF1α can bind and stabilize NICD, all together, promoting Notch signaling (Gustafsson et al., 2005b; Pistollato et al., 2010).

In canonic Notch signaling pathways, NICD binds to Rpbj to recognize specific promoter region elements, promoting or inhibiting the specific target genes (Castel et al., 2013). The canonic Notch target genes are Hes and Hey family genes. These genes belong to the bHLH family, with similar bioactivity, acting as transcriptional regulators (Iso et al., 2003). In different tissues, the main functional Notch downstream genes may be different due to their different target genes and specific regulations. In the brain, the main functional Notch downstream genes include Hes1, Hes6 and Hey1 (Lasky and Wu, 2005). In muscle, the genes that have been demonstrated to regulate muscle functions are Hes1 and Hey1 (Gustafsson et al., 2005b; Luo et al., 2005).

Notch signaling plays a key role in skeletal muscle development and regeneration. During the fetal development stage, continuously activating Notch in skeletal muscle can cause fetal fatality due to failure of normal skeletal muscle formation by inhibition of myogenesis. In adults, the function of Notch signaling is complicated. Previous research has demonstrated that muscle injury can upregulate the Notch ligand Delta in both activated satellite cells and in myofibers (Luo et al., 2005). Both of these upregulations indicate a promotion of Notch signaling. The upregulation of Delta in satellite cells is stimulated by an elevated NICD level. The increase of Delta in myofibers can subsequently activate Notch signaling in activated satellite cells. These results imply that the proliferation of activated satellite cells require activating Notch signaling. To confirm these results, the researchers demonstrated that inhibition of Notch signaling impeded satellite cell proliferation and impaired muscle regeneration. However, on the other hand,

constitutive Notch activation in satellite cells lead to an increase in Pax7 positive satellite cells but impaired muscle regeneration through inhibition of myogenic differentiation (Bi et al., 2016). Also, another study has demonstrated that, during asymmetric division, the self-renewal daughter cells exhibit higher Notch signaling levels while the differentiating daughter cells show significantly lower Notch signaling (Kuang et al., 2007). These results indicate that Notch signaling is helpful for self-renewal rather than differentiation. All these results imply that muscle injury requires accurately Notch regulation. When satellite cells need to proliferate, the Notch signaling should be elevated to promote proliferation and self-renewal. After that, Notch signaling should be inhibited to release the blocked myogenic differentiation.

Notch downstream genes, such as Hes1 and Hey1, belong to the bHLH family, which can recognize the E-box in the promoter region and can promote gene expression (Iso et al., 2003). By upregulated Notch signaling, Pax7 level can be significantly increased. Meanwhile, the myogenic factor Myod level is decreased.

It has been long known that hypoxia inhibits stem cells differentiation in different tissues, such as the brain and muscle (Gustafsson et al., 2005b). This kind of inhibition is similar to the function of activated Notch signaling that researchers have considered the relationships between these two important pathways. In tumors, the impact of Notch activating towards cancer cells shows significant similarities with hypoxia treatment (Yuan et al., 2015). These phenomena imply a close relationship between Notch signaling and hypoxia. Until recently, studies have demonstrated high relationships between Notch and hypoxia inducible factor 1 α (HIF1α). HIF1α can promote Notch signaling in two different ways. First, HIF1α can promote the function of γ-secretase

(Villa et al., 2014b). This kind of promotion does not rely on an increased transcription level. Rather, HIF1α can bind to γ-secretase to increase the activation of γ-secretase. This promotive effect is more dramatic after Notch receptors have bound to the ligands. The HIF1α induced activity of γ-secretase can be abolished by a γ-secretase inhibitor. Besides that, HIF1α can bind to the cleaved NICD to stabilize NICD and drive it to the nuclei (Gustafsson et al., 2005b). During hypoxia, HIF1α can be found enriched in NICD DNA binding regions. This means that HIF1α may participate in the transcriptional regulation of NICD. All these results indicate that there is a tightly related relationship between Notch and HIF1α. But besides HIF1α, the function of other hypoxia inducible factors with Notch is still unknown.

1.4.2   Wnt Signaling Controls Satellite Cells Expansion

Wnt signaling is very important in regulating the cellular process. So far it has been reported that Wnt can promote cell proliferation in multiple tissues, determine cell fate, participate in regulation of morphology and the subsequent cell attachment efficiency (Klaus and Birchmeier, 2008).

In the Wnt/β-catenin pathway, after receiving Wnt proteins from extracellular matrix by receptors named Frizzled, Wnt cascade pass through to β-catenin (MacDonald et al., 2009). The β-catenin level determines the final output. In normal conditions, the dynamic consistent levels of cytoplasmic β-catenin are kept by constitutive degradation by phosphorylation by GSK3-β (MacDonald et al., 2009). Then, phosphorylated β-catenin is bound by the ubiquitin ligase and degraded by the proteasome as normal protein degradation processes. The activation of GSK3-β can be inhibited by drugs designed for Wnt signaling inhibition. In physiological conditions, the activation of

GSK3-β can be inhibited by Dishevelled, which is activated by the binding of Wnt or Frizzled (Clevers and Nusse, 2012; Hayward et al., 2008; Naito et al., 2012). Free cytoplasmic β-catenin will increase after the activation of Wnt signaling. The released β-catenin binds with cofactors and transfers to the nucleus and interacts to promote or inhibit the target gene expression.

Like Notch signaling, the fact that Wnt signaling highly participates in satellite cell and myogenic differentiation has been demonstrated by increasing evidence (Brack et al., 2007; Cisternas et al., 2014; Jones et al., 2015; Majmundar et al., 2015; Otto et al., 2008a; Suzuki et al., 2015; Trensz et al., 2010). However, the exact roles of Wnt in satellite cell regulation is still controversial.

Nohno's group believed that Wnt signaling activation can terminate the stemness of muscle stem cells and induce myogenic commitment and differentiation (Tanaka et al., 2011). They compared the performance of muscle regeneration by either inhibiting or activating Wnt signaling and they noticed that satellite cells only respond to Wnt signaling at a late stage of myogenic differentiation (Tanaka et al., 2011). Inhibition of Wnt signaling can largely impede muscle regeneration by reducing the myofiber size due via decreased Myogenin levels. The activation group exhibited relatively better regeneration with an efficient repair and larger fiber size.

However, another group reported that β-catenin can promote satellite cell self-renewal and instead of inducing withdrawal from the cell cycle to commit to differentiation, it can protect satellite cells from myogenic differentiation (Perez-Ruiz et al., 2008). They found that overexpression of mutant β-catenin leads to downregulation of MyoD and Myogenin expression. Also, by inhibiting the activity of GSK-β, the

researchers achieved the same results. On the contrary, reduced β-catenin level in myoblasts can largely reduce self-renewal (Perez-Ruiz et al., 2008). However, the regulation of β-catenin towards Myod seems complicated in that another study demonstrated that β-catenin can bind with Myod directly to enhance the transcriptional activity of Myod (Kim et al., 2008).

Not only does this work as a short-term effect on growth factors participating in short-term cellular biology regulations, it also has been reported that the level of Wnt protein in skeletal muscle is highly related to long-term physiological changes such as aging (Brack et al., 2007). In aged mice skeletal muscle, Wnt family proteins are significantly higher than that in young mice. Also, studies have demonstrated that this high level of Wnt proteins is harmful for muscle regeneration due to reducing satellite cell expansion and self-renewal in vivo and in vitro. A high level of Wnt in skeletal muscle can not only impede muscle regeneration by inhibiting satellite cell expansion and self-renewal, but also it facilitates fibrosis (Brack et al., 2007). Non-efficient regeneration always induces fibrosis due to continuously activated immune response. Moreover, Wnt signaling can directly work on fibroblast progenitors and promote fibrosis. Altogether, the mechanism leads to impaired muscle regenerations in old mice compared with young mice by a high level of Wnt signaling. This kind of impairment can be rescued by Wnt signaling inhibitors. The inhibitors targeting β-catenin can inhibit downstream transcriptional activities.

The interesting thing about Wnt is that Wnt signaling always works together with Notch signaling (Hayward et al., 2008). This association is that Wnt signaling seems to work the opposite way with Notch (Tian et al., 2015). Notch can prevent satellite cells

from differentiation and keep satellite cells stemness, while Wnt signaling promotes satellite cells' differentiating commitment and facilitates differentiation and myotube fusion. Notch signaling activation can promote satellite cell proliferation and self-renewal, thereby contributing to the maintenance of satellite cell population. However, when activating Wnt signaling, satellite cells pretend to withdraw from the cell cycle and undergo myogenic differentiation. The competitive function of Wnt signaling and Notch signaling is very important in muscle physiological regulation. During muscle regeneration, satellite cells need to proliferate to produce myoblasts and keep stem cell population, which can be achieved by elevated Notch signaling and inhibited Wnt signaling. Afterwards, inhibition of Notch signaling cannot push the myoblasts into mature myocytes, thus activated Wnt signaling is required for myogenic commitment and myogenic differentiation. After regeneration, Wnt signaling should be downregulated for the maintenance of stem cell stemness. Likewise, only block Wnt signaling cannot promote stem cell self-renewal, thus activated Notch signaling is needed. Having balance and accurate levels of Notch and Wnt is very important for efficient muscle regeneration. Either too high or too low level of Notch and Wnt can lead to impaired muscle regeneration. It has also been reported that NICD can directly bind with β-catenin and can regulate the transcriptional activity of β-catenin (Kwon et al., 2011). However, whether NICD can promote or inhibit Wnt signaling is not exclusively dependent on NICD; instead, it depends on the whole cellular condition (Kwon et al., 2011).

In addition to Notch signaling, Wnt signaling can also cross talk with hypoxia-inducible factors. In cancer cells, it has been reported that HIF1α can bind with β-catenin and can inhibit transcriptional activity, leading to the inhibition of Wnt signaling (Kaidi

et al., 2007). In satellite cells, it has also been reported that, during ischemic injury, which can induce skeletal muscle hypoxia, the Wnt signaling is largely been reduced (Majmundar et al., 2015). By knocking out HIF1α or supplementing the Wnt agonist, the inhibition of myogenic differentiation can be rescued. This result indicates that hypoxia can inhibit Wnt signaling by HIF1α through β-catenin. However, the direct binding of β-catenin and HIF1α is not demonstrated (Majmundar et al., 2015).

Even though the relationship of hypoxia and Wnt is quite clear, the cross talk of Wnt and Notch under hypoxia conditions is still less known. Given the fact that Notch signaling can regulate Wnt differently under different conditions, there is a possibility that Notch may regulate Wnt differently under a hypoxia state. More studies need to be done in this area.

1.4.3   TGF-β Signaling Controls Skeletal Muscle Mass

Different from the regulation of determinations on satellite cell differentiation, quiescence or self-renewal, which is mainly controlled by transcriptional regulators such as Pax7 or Myod, muscle mass is mainly controlled by the balance of protein synthesis and degradation (Mitch and Goldberg, 1996; You et al., 2015). Excessive synthesis induces muscle hypertrophy, while excessive degradation leads to muscle atrophy. It is widely accepted that the key regulation mechanism of protein synthesis in muscle is the Akt/protein kinase B (PKB)–mammalian target of rapamycin (mTOR) signaling pathway axis (Egerman and Glass, 2014; Sartori et al., 2014; You et al., 2015). This pathway controls the protein synthesis tools, S6 and eukaryotic translation initiator factor 4E (eIF4E). Meanwhile, it regulates the inhibitor of this tool, 4E-binding protein 1 (4EBP1) (Haghighat et al., 1995; Hara et al., 1997). For protein degradation, the direct functional

factors are Fbxo32 and MuRF1, which are the two most important E3 ubiquitin ligases participating in muscle degradation (Bodine et al., 2001; Gomes et al., 2001; Mammucari et al., 2007). Both of these two genes are regulated by FoxO3, which can be inhibited by Akt. Thus, it is in this way that the Akt-mTOR pathway controls muscle mass.

The TGFβ superfamily consists of over 30 factors. All these factors are secreted extracellularly, which can be recognized by specific receptors on the cell surface (Shi and Massague, 2003). After the binding of extracellular secretory factors with four main types of receptors, the secondary messengers, called SMADs, are phosphorylated and dimerized followed by a transfer to the nucleus (Massague, 2012). The subsequent message transfer is similar, but has some differences. Due to different activated transcriptional regulators, the output is largely different. Activins, Myostatin and GDF8 can be recognized by TGF-βR1 and activates SMAD2/SMAD3. Follistatin can be recognized by TGF-βR1, ALKs, and results in activation of SMAD2/SMAD3 or SMAD1/SMAD5/SMAD8. BMPs and GDF can be recognized by BMPRII, and also can activate SMAD2/SMAD3. Due to SMAD2/SMAD3 being phosphorylated by multiple cell surface receptors, the functional efficiency of different TGF-β factors can be regulated by competitive mechanisms (Massague, 2012).

As mentioned previously, the muscle mass core regulation pathway is through the Akt pathway. The factors and mechanisms that can connect TGF-β and Akt is that phosphorylated SMAD2/SMAD3 can inhibit the activation of Akt (Zhang et al., 2013). Also, SMAD2/SMAD3 can directly inhibit the function of MuRF1 and MAFbx (Sartori et al., 2013). Thus, activation of SMAD2/SMAD3 can inhibit excessive Akt activation, increase muscle protein degradation and keep physiological normal muscle mass. The

most significant factor among TGF-β involved in muscle mass is Myostatin (Kambadur et al., 1997; McPherron et al., 1997). Normal levels of Myostatin can inhibit excessive muscle protein synthesis. Myostatin-deficient animals develop obvious muscle hypertrophy, which is conserved in different animals, such as mice, bulls and humans. Myostatin mice exhibit larger myofiber size but not myofiber number (McPherron et al., 1997). Besides the loss of secretory factor function, receptor functional deficiency or over functionality can also induce abnormal muscle mass. Overexpression of ALK4 and ALK5 can increase the phosphorylation of SMAD2/SMAD3, which can induce muscle atrophy (Kemaladewi et al., 2012).

Recently, new discoveries have demonstrated that BMP pathways highly participate in regulation of muscle mass. The core factor involved in this regulation is SMAD4 (Sartori et al., 2013). SMAD4 can interact with SMAD2/SMAD3 or SMAD1/5/8. Thus, SMAD2/3 competes with SMAD1/5/8 for interactions with SMAD4. The function of SMAD4 seems to only depend on inhibition of protein degradation by inhibition of Fbxo30, as suggested by the dramatically decreased muscle mass after denervation in SMAD4 deficient mice as compared to normal mice. SMAD1/5/8 can be activated by BMP7, which can explain how overexpression of BMP7 can help to preserve muscle mass after denervation (Sartori et al., 2013). This theory can also explain the function of Myostatin and how a Myostatin deficiency induced low level of SMAD2/3 can release more available SMAD4 for subsequent binding with SMAD1/5/8. Differently, the binding of SMAD4 with SMAD1/5/8 or SMAD2/3 can induce different gene expression, resulting totally different consequences.

The regulation of TGF-β signaling was reported to show cross talk with hypoxia. It has been reported that TGF-β1 induces HIF1α stabilization through selective inhibition of PHD2 expression (McMahon et al., 2006). The canonical mechanism of stabilization of HIF1α is through the inhibition of PHD2 activation. PHD2 can hydroxylate HIF1α and commit the protein degradation process. By inhibiting PHD2, avoiding tagged HIF1a can be stabilized. However, TGF-β stabilized HIF1a through decreasing the protein level of PHD2 through the inhibition of transcription. This means that HIF1α can be stabilized even in sufficient oxygen conditions. On the other hand, HIF1α can selectively inhibit the function of BMPs (Pistollato et al., 2009; Tseng et al., 2010). HIF1α deficiency can increase the phosphorylation of SMADs, which indicates that HIF1α can inhibit BMP signaling activity even though the mechanism of HIF1α is still unknown. The HIF-TGF□ in muscle is still less documented, but the close relationship has been demonstrated in other tissues, which may imply an important function in muscle.

1.4.4 JAK-STAT Signaling Controls Satellite Cell Expansion

The Janus kinase (JAK)–signal transducer of activators of transcription (STAT) pathway is an evolutionarily conserved signaling pathway, which participates in different kinds of cellular regulation, such as differentiation, proliferation, apoptosis, oncogenesis and immune reactions (O'Shea et al., 2015; Rawlings et al., 2004). As a cell signaling pathway, JAK-STAT pathway not only works solely, but it can also cross talk with different signaling pathways. Thus, when talking about regulations of hypoxia in satellite cells and muscle homeostasis, the JAK-STAT pathway is a very important participant that can't be ignored.

The JAK-STAT signaling pathway consists of three main components: a cell surface receptor, a phosphorylation kinase JAK and a transcriptional regulator, STATs (Aaronson and Horvath, 2002). The binding of different ligands in the extracellular matrix, such as interferon, interleukin or growth factors, with specific cell surface receptors, activates the kinase activity of associated JAKs. The activated JAKs recruits and phosphorylates STATs (O'Shea et al., 2015). Phosphorylated STATs are activated and form hetero or homo dimers. Dimerized STATs translocate to the cell nucleus and regulate gene expression. Besides STATs, JAKs can also phosphorylate other substitutes, which can compete with STATs. For example, SOCS can bind and inhibit the activated JAKs and block the recruitment and phosphorylation of STATs (Croker et al., 2008).

Although the regulation of the JAK-STAT pathway looks simple, it participates and regulates multiple pathways by the combination of two JAKs and seven STATs. Binding growth hormone factors to their specific receptors can phosphorylate JAK2, followed by the recruitment and activation of STAT5 or STAT1. On the other hand, IFNα/β can activate STAT1/2, while IFN-γ can only activate STAT1 (Ho and Ivashkiv, 2006; Ivashkiv and Donlin, 2014; Miscia et al., 2002; Qing and Stark, 2004).

In muscle, it has been reported that the inhibition of JAK-STAT signaling stimulates adult satellite cell function (Price et al., 2015; Tierney et al., 2014). It has been well-known that muscle regeneration efficiency of aged mice is much lower than that in younger mice. However, the reason why aged mice exhibit worse regeneration efficiency is still not clear. By comparing freshly isolated satellite cells from aged mice and young mice using global gene expression analysis, researchers have found a much higher level of JAK-STAT target genes and JAK-STAT coactivators in aged mice, indicating an

elevated JAK-STAT signaling level during aging (Price et al., 2015). They also checked the phosphorylated STAT3 level and found a higher level of pSTAT3, validating the increase of JAK-STAT signaling. By the inhibition of JAK2 or STAT3 using siRNAs, the proliferation defect of satellite cells in cultured myofiber from aged mice was rescued (Tierney et al., 2014). After reducing JAK2 or STAT3 expression levels, the engraftment of myoblasts was largely enhanced. Also, injection of JAK-STAT inhibitors can enhance the muscle regeneration in vivo. All these results indicate that inhibition of the JAK-STAT pathway, specifically the JAK2-STAT3 pathway, can rescue aging-induced impaired muscle regeneration.

At the same time, another group independently reported the function of STAT3 in satellite cell regulations from another aspect. It was reported that STAT3 promoted myogenic lineage progression and inhibited self-renewal and proliferation, which is similar to the previous report (Tierney et al., 2014). In this case, the elevated pSTAT3 level was found to correspond with the level of IL-6. Both in muscle wasting and during aging, IL-6 is largely increased. As a downstream factor of IL-6, constitutive activation of STAT3 can impair the proliferation and self-renewal of satellite cells, impairing muscle regeneration. However, long-term inhibition of STAT3 also impeded muscle regeneration by inhibiting myogenic differentiation. But, in the early stages of regeneration, the short-term decrease of STAT3 is helpful for muscle regeneration, and results from an increased proliferation of satellite cells. Thus, having an accurate level of STAT3 is very important in muscle satellite cell homeostasis (Tierney et al., 2014).

Hypoxia can control the level of STAT3 in different manner. It has been reported that hypoxia can induce a decrease in SOCS3 (Toth et al., 2011). SOCS3 compete with

STAT3 with JAK (Croker et al., 2008). Thus, by inhibition of SOCS3, STAT3 can be activated. Also, hypoxia can induce the production and release of IL-6 (Toth et al., 2011). IL-6 can largely promote STAT3 activity. From a general biological view, STAT3 can promote satellite cell differentiation and inhibit proliferation. However, hypoxia can induce opposite changes, such as inhibited differentiation and promoted self-renewal. This makes the regulation mechanism between hypoxia and STAT3 more complicated. More research is needed in this area.

1.5 Cross Talk between Muscle and Other Tissues

During muscle regeneration, the oxygen level in muscle microenvironments changes dynamically due to the formation of new vessels and increased oxygen expenditure. In the early stages of injury, oxygen transport and availability decreases compared with normal conditions due to the break of blood vessels (Merrick, 2002). Meanwhile, muscle regeneration requires more energy, protein and oxygen. The imbalance of oxygen availability and requirement increases a hypoxia reaction in the muscle. The hypoxic challenge is not only faced by myogenic lineage cells, but also other types of cells that may regulate the regeneration process.

The connection between the muscle and the immune system is the most important regulation mechanism amongst all these cross talks. The immune cells travel around the whole body, preventing invasions of bacteria and viruses, cleaning out dead cells and debris and secreting cytokines to regulate cellular behaviors (Castelo-Branco and Soveral, 2014; Hansen et al., 2015). During muscle regeneration, immune cells invade the muscle through the broken vessels, cleaning dead fibers and engulfing debris. At the same time, they release cytokines to regulate muscle regeneration directly and indirectly

(Saini et al., 2016; Tidball and Villalta, 2010). As mentioned previously, IL-6 secreted from the macrophages can activate STAT3 in satellite cells and promote myogenic differentiation. Also, IL-4 and IL-13 can promote the proliferation and inhibit the adipogenic differentiation of FAPs cells resident in the muscle tissue (Heredia et al., 2013). IL-4- and IL-13-activated FAPs cells can engulf dead myofibers and debris, secreting Wnt beneficial to muscle regeneration (Heredia et al., 2013; Joe et al., 2010). The release of IL-4, IL-6 and IL-13 are highly regulated by the oxygen level. It has been demonstrated in different types of cells that hypoxia can induce the expression of IL-6. On the other hand, IL-4 can increase the expression of hypoxia-inducible factors. Even though the relationship between immune factors and muscle under a hypoxia state has not been determined, there should be a complex regulation between hypoxia, the immune system and muscle regeneration.

Different from the previous view, skeletal muscle is no longer just considered as a motive organ. Muscle-released factors participate in the regulation of other tissues, such as Irision for brown adipose tissue and Cathepsin B for the brain. The interesting fact is that the release of these myokines can be induced by exercising the muscle. But precisely how exercising can induce the release of myokines is unknown. One possibility is that the release of myokines is regulated by oxygen level when doing exercise, and the dynamic changes in oxygen levels stimulate the secretion. More research needs to be done to investigate the principal mechanism.

1.6 Hypoxia: Not Just Low Oxygen Level

1.6.1 1.6.1 Hypoxia-Related Factors

The fluctuating oxygen in cells not only regulates the energy supply by changing the ratio of respiration chains in mitochondria, but it also induces other molecular changes. There are two main types of hypoxia-related factors, the reactive oxygen species (ROS) and hypoxia inducible factors (HIFs) (Clanton, 2007; Sabharwal and Schumacker, 2014; Semenza, 2014).

ROS is reactive oxidative species. ROS is not only induced by hypoxia, but also other environmental stresses, such as overheating and ultraviolent exposure (Circu and Aw, 2010). Continuous high levels of ROS can induce cell damage and apoptosis. ROS participates in many cellular regulations, such as proliferation, differentiation, immune reactions, morphology changes and apoptosis (Circu and Aw, 2010; Day and Suzuki, 2005; Gauron et al., 2013). It has been reported that the accumulation of ROS can immediately downregulate cyclin D1, inducing a cell cycle arrest in the G2 phase (Verbon et al., 2012). ROS induces DNA damage by promotion of p53 and activation of poly (ADP-ribose) polymerase RARP, leading to an apoptosis cascade (Montero et al., 2013). ROS can also regulate proliferation and survival through MAPK, PI3K and PTEN (Morgan and Liu, 2011; Son et al., 2013). There is an interactional regulation between ROS and NF-kB where ROS can inhibit or promote NF-kB signaling, while the downstream genes of NF-kB can regulate the generation of ROS (Morgan and Liu, 2011). All together ROS is a very important factor that is involved in the reaction of the hypoxia challenge.

Another big family of hypoxia-related factors are HIFs. HIFs are transcription factors consisting of two main subunits, the oxygen sensor subunit HIFα and the oxygen

insensitive group HIFβ (aryl hydrocarbon receptor nuclear translocator, Arnt) (Jaakkola et al., 2001; Semenza, 2003). These two factors bind with each other through Per-Arnt-Sim (PAS) domains, forming a heterodimeric transcriptional regulator. The heterodimer forms a basic helix-loop-helix (bHLH) domain, regulating target gene transcription through C-terminal transcriptional transactivation domains (TADs) (Wang et al., 1995). The activation of HIF signaling is controlled by oxygen level by HIFα degradation. At an oxygen concentration above 5%, HIFα can be hydroxylated by prolyl hydroxlase domain proteins (PHD) at two specific proline residues (Weidemann and Johnson, 2008). The tagged HIFα can be recognized by von Hippel-Lindau tumor suppressor protein (Vhl), initiating the ubiquitin degradation cascade (Ivan et al., 2001; Maxwell et al., 1999; Wang et al., 1995). When challenged by hypoxia, the hydroxylation rate is reduced and induces accumulation of HIFα. Besides a decreased oxygen level, inhibition of activation of PHD or ablation of Vhl can also lead to stabilization of HIFα (Matsuura et al., 2011; Watanabe et al., 2014). Stabilized HIFα-forming heterodimer with HIF1β, translocating to the nucleus regulates gene expression (Semenza, 2003; Semenza and Wang, 1992). In addition, the factor inhibiting HIF (FIH) hydroxylates an asparagine residue in the TAD, blocking binding of the transcriptional coactivator CBP/p300, which negatively regulates HIF signaling (Mahon et al., 2001).

There are three types of HIFα that have been identified: HIF1α, HIF2α and HIF3α (Dengler et al., 2014; Greer et al., 2012; Semenza, 2012). HIF1α and HIF2α function similarly by forming a heterodimer with Arnt (Simon, 2008). However, HIF3α acts in a completely opposite manner. HIF3α consists of three isoforms, Hif3α, neonatal and embryonic PAS (NEPAS), and an inhibitory PAS protein (IPAS). HIF3α and

NEPAS do not have a transcriptional regulating activity, even when binding with Arnt. This binding process will compete with HIF1α and HIF2α at the Arnt binding sites, which can finally inhibit the transcriptional activity of HIF1α and HIF2α (Li et al., 2006). Different than HIF3α and NEPAS, IPAS do not bind with Arnt. However, IPAS binds with HIF1α and HIF2α, thereby preventing the binding of HIF1α and HIF2α with Arnt (Weir et al., 2011). All together the three HIF3α isoforms act as negative regulators of HIF signaling.

The heterodimer of HIF and Arnt cooperates with CBP/p300, forming a bHLH transcriptional regulator, which can specifically recognize hypoxia-responsive elements (HRE) in DNA and can regulate the transcription of target genes. HIFs target genes participate in different cell behaviors, such as p53 for survival and apoptosis, VEGF for angiogenesis, MMP for extracellular matrix regulation and so on (Greer et al., 2012). Recently, it has been found that HIF1α can act as post-transcriptional regulators. For example, in tumor cells, HIF1α has demonstrated to bind with γ-secretase and increase its activation (Villa et al., 2014a). Also, HIF1α can stabilize NICD after cleavage, promoting notch signaling (Gustafsson et al., 2005a). In terms of Wnt, HIF1α can bind with β-catenin, inhibiting Wnt signaling, even though the mechanism is still unknown (Kaufman, 2010).

1.6.2 Cross Talk between HIF and Other Cell Signaling

HIF is involved in different cell behaviors and metabolism by its interaction with different cell signaling, such as cell fate determination by Notch and Wnt, proliferation by Cyclin D1, fibrosis by TGF-β and apoptosis by p53.

It has been found, in different tissues, that HIF can promote Notch in several different ways. The first time a researcher found the interaction of HIF and Notch was in neurons and muscle progenitor cells (Gustafsson et al., 2005b). It was reported that hypoxia blocked neuronal and myogenic differentiation in a Notch-dependent manner. Hypoxia activates Notch-responsive promotors and enhances the expression of Notch target downstream genes. Also, HIF1α can interact with NICD to stabilize it and drive it to the nucleus. HIF1α was found to be recruited to the Notch-responsive promoter region to promote Notch target genes, indicating its function of elevating Notch. Inhibition of Notch signaling can rescue the hypoxia-induced blockage of differentiation. All these results, at the cell and molecular levels, provide the first evidence that HIF1α can inhibit myogenic and neuronal differentiation (Gustafsson et al., 2005b). Later, the interaction of HIF1α and Notch signaling in regulating tumor metabolism has been demonstrated in different kinds of tumors, such as medulloblastoma and glioblastoma (Pistollato et al., 2010). The mechanism of how HIF mediates Notch has been further studied. In addition to binding with NICD, HIF1α can also interact with γ-secretase and enhance its activity (Villa et al., 2014b). Specific γ-secretase inhibitors can inhibit HIF1α-induced promotion of Notch. All these results demonstrate that by increasing and stabilizing NICD, HIF1α can promote Notch signaling. More recently, it has been shown that HIF1α can promote Notch signaling independent of Notch ligand and Notch receptor binding (Mukherjee et al., 2011). In crystal cells, HIF1α co-localized with endocytic Notch receptors and stabilized NICD during cleavage, transferring NICD to the nucleus to perform its transcriptional regulation.

Different from the function of HIF on Notch, the reaction between HIF and Wnt has been inconsistent between different studies. Some studies showed a promoting Wnt function of HIF1α, while other studies demonstrated a completely opposite result (Kaufman, 2010; Majmundar et al., 2015; Mazumdar et al., 2010). In embryonic neuron stem cells and hepatocellular carcinomas, HIF1α can activate β-catenin and increase the downstream genes of Wnt signaling (Kaufman, 2010; Mazumdar et al., 2010). In hypoxia states, the researchers noticed an increased level of Wnt signaling by quantifying the Wnt downstream regulator protein level. The promoted Wnt signaling can be abolished by knocking out HIF1α. The researchers concluded that the hypoxia-induced promotion of Wnt is dependent on HIF1α (Mazumdar et al., 2010). In osteoblast and myoblast, however, it has been confirmed that HIF1α can strongly inhibit Wnt signaling through a different mechanism (Chen et al., 2013; Majmundar et al., 2015). Studies have demonstrated that osteoblast growth is inhibited under hypoxia through the cooperation of HIF-1α with Osterix (Osx) to inhibit the Wnt pathway. In myoblasts, HIF1α inhibits the activation of β-catenin, inhibiting Wnt signaling and blocking late stage myogenic differentiation (Majmundar et al., 2015). All these results imply that HIF1α may cooperate with some other factors that haven't been discovered yet and these cooperators may or may not lead to dramatically different results. However, this is just one possible explanation for these conflicting results; more studies need to be done in the future.

Besides these two pathways HIF is involved in and mediates, HIF can also cross talk with other pathways in a converse direction. Recently, it was reported that the basal level of HIF mRNA and the protein level is controlled by NF-kB signaling (Gorlach and Bonello, 2008; van Uden et al., 2008). Researchers have found that the NF-kB subunits

p50 and p65 can directly interact with HIF1α by transcriptional recognition of NF-kB at the promoter region of HIF1α at about -197/188 bp (Yoshida et al., 2013). Mutation of this site will block the impact of NF-kB towards HIF1a. More importantly, the specific NF-kB recognizing site in HIF1α is conserved among different species. Additional results confirmed the regulation function of NF-kB in the HIF pathway and that through a knock out or genetic deficiency of IKKα and IKKβ, the mRNA and protein level of HIF1α won't change. All these results demonstrate that NF-kB can transcriptionally regulate the expression level of HIF1α (Yoshida et al., 2013).

In physiological conditions, organs need to maintain normal function under dynamically consistent levels of oxygen. Thus, theoretically and practically, HIF can cross talk with different pathways to participate in cellular behavior regulations.

1.6.3   The Impact of Hypoxia in the Embryonic Development Stage

During the embryonic development stage, animals are challenged by a long-term low oxygen level condition due to the lack of blood vessels and a restricted rate of oxygen diffusion (Dunwoodie, 2009). However, this long-term hypoxic condition is usually harmful in adults. The HIF pathway is involved to increase the tolerance of, or rescue from, pathological hypoxia. However, this low oxygen level in the embryonic stage is necessary for normal fetal development. Hypoxic microenvironments create specific niches regulating cellular differentiation and the HIF system contributes to the regulation of differentiation and morphogenesis. In the 1970s, researchers demonstrated that a normal neural fold was dependent on a low O2 level culture condition ex utero (Morriss and New, 1979). The hypoxic condition in the embryonic development stage was confirmed in 2003, where it was discovered that pimonidazole and EF5 can bind

with DNA and proteins only in hypoxic conditions (Bruick, 2003). Further, genetic manipulation of HIF deficiency models in various species reveals the importance of HIF proteins in embryos (Simon et al., 2002).

Oxygen availability controls the development of the blood circulation system, which is responsible for oxygen delivery. This oxygen delivery system should ensure that resident cells maintain proper metabolic activity. During vascular development, vascular endothelial growth factor (VEGF) is a major angiogenic growth factor, which is highly controlled by oxygen level (Jarecki et al., 1999). Similar to VEGF, other vascular formation growth factors, such as TGFβ and FGF, are also mediated by HIF (Caniggia et al., 2000; Shweiki et al., 1992). When cells are challenged by hypoxia, they release more angiogenic factors, which attract vascular endothelial cells to migrate to the oxygen-starved cells. This theory has been confirmed by the Arnt deficient mice model. Knocking out of Arnt induces fetal lethality in E10.5 due to a deficiency of vascular formation (Adelman et al., 1999). The initial vascular beds showed normal development; however, the vessel remodeling failed to process. Consistent with the phenotype, the VEGF mRNA and protein level in embryos is significantly lower in Arnt deficient mice, which means Arnt can regulate VEGF level.

Besides the blood circulation system of the developmental fetus, the development of the placenta is also impacted by oxygen concentration. In mice, the embryo undergoes glycolysis for energy due to lack of oxygen until the circulation system formation in the placenta around E11 (Kozak et al., 1997). The establishment of placental circulation is controlled by oxygen sensor regulators. Arnt or HIF deficient mice exhibit abnormal development of the placenta (Adelman et al., 2000; Cowden Dahl et al., 2005; Gnarra et

al., 1997). Excessive accumulation of HIF by deletion of Vhl and PHD also induces aberrant placental architecture (Gnarra et al., 1997).

Global knock out of HIF family genes can induce early fetal lethality. However, single deficiency in one of the HIF family genes (except for HIF1α, which can also induce fetal lethality) may also cause mutant mice. Knocking out HIF2α allows for birth, even though the neonatal mice will die soon after birth (Compernolle et al., 2002). HIF2α deficient mice exhibited severe cardiovascular disease, which is different from the HIF1α deficient phenotype. This result implies a common function of HIF1α and HIF2α, though they have their own specific roles (Simon, 2008).

In addition to oxygen delivery-related organs, oxygen level can also affect bone morphogenesis. The growth of bone needs a well-programmed cascade proliferation, differentiation and apoptosis (Raggatt and Partridge, 2010). As the bone grows thicker, more vessels need to be attracted to new bones. Thus, in the proliferating stage, osteoblasts and chondrocytes produce a low level of VEGF but a high level of collagen II, while in differentiated cells they release significantly higher levels of VEGF. Specific deletion of HIF1α in chondrocytes induces chondrocytes death, exhibiting obviously shorter limbs (Rajpurohit et al., 1996). This result demonstrates a crucial function of HIF1α in chondrogenesis.

In adipogenesis, HIF1α acts as a suppressor of adipogenic differentiation. Hypoxia inhibits adipogenesis through inhibition of the expression of peroxisome proliferative activated receptor-γ (PPARγ) (Yun et al., 2002). Ppar-γ is the critical transcriptional regulator of adipogenesis, which initiates the adipogenic cascade (Spiegelman, 1998). Knocking out HIF1α in fibroblasts can rescue the inhibition of hypoxia-induced

adipogenic differentiation (Yun et al., 2002). HIF1α inhibits Ppar-γ through induced expression of DEC1, a repressor of the Ppar-γ promoter. All these results demonstrate a necessary importance of hypoxia and HIF in embryonic development.

1.7   Muscle Hypoxia

1.7.1   Hypoxia Inhibits Myogenic Differentiation

Skeletal muscle distinguishes itself by its outstanding tolerance of dynamic changes of oxygen level due to the frequently changed oxygen level, which is highly dependent on the activity frequency. However, the chronic hypoxia impeded myogenesis is marked by the decreased fiber size of regenerated myofibers in ischemic conditions (Blaisdell, 2002). Long-term exposure in high altitudes also induces a smaller myofiber size and decreases the whole muscle size (Howald and Hoppeler, 2003). Given all this evidence, it is proposed that hypoxia can inhibit myogenic differentiation and alter the myoblast behavior.

A preliminary study demonstrates that, under as low as a 2% oxygen level, myogenic differentiation was inhibited (Yun et al., 2005). The strongest inhibition was found in a 0.01% oxygen level. Further, the study confirmed that the low differentiation rate is not due to a higher level of apoptosis. These results indicate that hypoxia inhibits myogenic differentiation by regulating the myoblast differentiation cascade (Yun et al., 2005).

The fact that hypoxia can inhibit myogenic differentiation has been widely confirmed; however, the explanations of the mechanism differ among studies (Gustafsson et al., 2005b; Majmundar et al., 2012; Yun et al., 2005). Some researchers have demonstrated that the hypoxia-induced inhibition of myogenic differentiation is

dependent on Notch, while others report that hypoxia won't alert Notch signaling in

myoblasts, even though the inhibition is independent of HIF1α. Even though the original

initial trigger of myogenic inhibition is contrasted among various studies, the reduced

level of the Myod protein has been identified by different studies. However, the reason

why the Myod protein level reduced is also conflicting among different groups. One

group believed that the repressed Myod level is induced by the active inhibition of Myod

transcription (Yun et al., 2005) and this inhibition fact has a transient impact that can be

recovered by long-term exposure in a 2% or lower oxygen level. This means that

myoblasts can adapt to a low oxygen environment. In another study, the researchers

demonstrated that the decrease of the Myod protein level is due to the accelerated

degradation rate of Myod proteins (Di Carlo et al., 2004) and the decrease in Myod

proteins can further decrease the Myod mRNA level, which makes the amount even less.

To prove their results were correct, the researchers transfected Myod into C3H10T1/2

cells. If the transfected cells were able to undergo myogenic normal differentiation, then

hypoxia should repress the transcriptional level of Myod first, otherwise it should be the

regulation of Myod protein post-transcriptional modification. Indeed, they identified an

inhibited differentiation in the Myod-transfected C3H10T1/2 cells. This result means that

hypoxia can directly modify the Myod protein. Then, they investigated the exact cause

that induced the decrease of the Myod protein. They asked if the Myod protein synthesis

rates dramatically decreased or if the Myod protein degradation increased a lot. To test

this question, they treated the C2C12 cells with a protein synthesis inhibitor,

cycloheximide. The inhibition of protein synthesis was confirmed by the decreased

protein level. After treating the cells for one hour with cycloheximide, they found a

dramatic decrease of Myod protein in the hypoxia treated group. This means the influx of

the Myod protein will not alert the protein level. Further, they tested the protein

degradation rates by treating the cells with protein degradation inhibitors. Interestingly,

the protein level maintained no difference between the hypoxia group and the control

after the protein degradation inhibitor treatment. All these results indicated that the

hypoxia-induced decrease of the Myod level was due to the increased protein degradation

rate (Di Carlo et al., 2004).

Even though the reason why hypoxia decreased the Myod level has been

demonstrated, the reason why hypoxia can induce an accelerating degradation of Myod is

still unknown.

1.7.2   Hypoxia Inhibits Myogenic Differentiation through Bhlhe40

Besides decreasing the level of Myod proteins, hypoxia can also inhibit myogenic

differentiation through other factors. Among these factors, Bhlhe40 has demonstrated to

be a significant contributor to the hypoxia-induced inhibition of myogenic differentiation.

Bhlhe40 belongs to the bHLH family and mediates multiple cellular behavior,

such as cell proliferation, apoptosis, adipogenesis and myogenesis (Honma et al., 2002;

Li et al., 2002; Liu et al., 2013). The function of Bhlheo40 in myogenic differentiation is

controversial and dependent on the different stages and pathways in which it is involved.

It has been reported that overexpression of Srebp-1α in myotubes can downregulate

muscle specific genes (Lecomte et al., 2010). Global gene expression profiles have

demonstrated that Bhlh40 is a target gene of Srebp-1α, as indicated by a dramatic

decrease in expression level in Bhlh40 overexpressed myotubes. Together with the fact

that 34% of genes inhibited by Srebp-1α can also be inhibited by overexpression of

Bhlh40, researchers have concluded that Srebp-1α may inhibit myoblast differentiation through Bhlh40. Further studies have demonstrated that overexpression of Bhlh40 can inhibit myoblast differentiation and promote skeletal muscle atrophy. Thus, it was concluded that Bhlh40 can inhibit myoblast differentiation mediated by Srebp-1α (Lecomte et al., 2010). However, another study group demonstrated some controversial results. The skeletal muscle development exhibits no statistical differences between Bhlh40 mutant and normal mice. However, during muscle regeneration, Bhlh40 deficient mice showed impaired muscle regeneration with enhanced myoblast proliferation and inhibited differentiation (Sun et al., 2007). Also, in vitro results demonstrated that Bhlhe40 deficiency significantly inhibited myogenic differentiation. Constitutive expression of Bhlhe40 can rescue the increased proliferation and decreased differentiation. The mechanism through which Bhlhe40 deficiency inhibits myogenic differentiation is that Bhlhe40 can inhibit Notch signaling. Without Bhlhe40, Notch signaling is consistently highly activated in myoblasts and inhibits myogenesis (Sun et al., 2007; Wang et al., 2015).

The microarray analysis of hypoxia-induced genes in primary myoblasts demonstrated that Bhlhe40 was highly promoted after hypoxia treatment (Wang et al., 2015). Together with the fact that overexpression of Bhlhe40 can inhibit the differentiation of myoblasts and the knock down of Bhlhe40 can promote myogenic differentiation, the author hypothesized that hypoxia can inhibit myogenic differentiation through mediating Bhlhe40. Further results confirmed this hypothesis that inhibition of Bhlhe40 can partly rescue myogenic differentiation under hypoxia. Thus, the author concluded that hypoxia can inhibit myogenic differentiation through Bhlhe40. The

interesting part is that the promotion of Bhlhe40 induced by hypoxia is independent of

HIF1α. Instead, the elevated Bhlhe40 is dependent on the increased p53 level. This result

implies that p53 can be promoted by hypoxia independent of HIF1α (Wang et al., 2015).

1.8 References

Aaronson, D. S. and Horvath, C. M. (2002). A road map for those who don't know JAK-STAT. *Science* **296**, 1653-1655.

Adelman, D. M., Gertsenstein, M., Nagy, A., Simon, M. C. and Maltepe, E. (2000). Placental cell fates are regulated in vivo by HIF-mediated hypoxia responses. *Genes Dev* **14**, 3191-203.

Adelman, D. M., Maltepe, E. and Simon, M. C. (1999). Multilineage embryonic hematopoiesis requires hypoxic ARNT activity. *Genes & Development* **13**, 2478-2483.

Allen, R. E. and Boxhorn, L. K. (1987). Inhibition of skeletal muscle satellite cell differentiation by transforming growth factor-beta. *Journal of Cellular Physiology* **133**, 567-72.

Amthor, H., Sartori, R., Schirwis, E., Blaauw, B., Bortolanza, S., Zhao, J., Enzo, E., Stantzou, A., Mouisel, E., Toniolo, L. et al. (2013). BMP signalling controls muscle mass. *Neuromuscular Disorders* **23**, 850-851.

Andersson, E. R., Sandberg, R. and Lendahl, U. (2011). Notch signaling: simplicity in design, versatility in function. *Development* **138**, 3593-3612.

Asakura, A., Komaki, M. and Rudnicki, M. A. (2001). Muscle satellite cells are multipotential stem cells that exhibit myogenic, osteogenic, and adipogenic differentiation. *Differentiation* **68**, 245-253.

Astrup, A., Bulow, J., Madsen, J. and Christensen, N. J. (1985). Contribution of BAT and skeletal muscle to thermogenesis induced by ephedrine in man. *Am J Physiol* **248**, E507-15.

Beauchamp, J. R., Heslop, L., Yu, D. S., Tajbakhsh, S., Kelly, R. G., Wernig, A., Buckingham, M. E., Partridge, T. A. and Zammit, P. S. (2000). Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells. *Journal of Cell Biology* **151**, 1221-34.

Ben-Yair, R. and Kalcheim, C. (2005). Lineage analysis of the avian dermomyotome sheet reveals the existence of single cells with both dermal and muscle progenitor fates. *Development* **132**, 689-701.

Berkes, C. A. and Tapscott, S. J. (2005). MyoD and the transcriptional control of myogenesis. *Seminars in Cell & Developmental Biology* **16**, 585-595.

Bi, P., Yue, F., Sato, Y., Wirbisky, S., Liu, W., Shan, T., Wen, Y., Zhou, D., Freeman, J. and Kuang, S. (2016). Stage-specific effects of Notch activation during skeletal myogenesis. *Elife* **5**.

Bi, P. P., Shan, T. Z., Liu, W. Y., Yue, F., Yang, X., Liang, X. R., Wang, J. H., Li, J., Carlesso, N., Liu, X. Q. et al. (2014). Inhibition of Notch signaling promotes browning of white adipose tissue and ameliorates obesity. *Nature Medicine* **20**, 911-918.

Blaisdell, F. W. (2002). The pathophysiology of skeletal muscle ischemia and the reperfusion syndrome: a review. *Cardiovasc Surg* **10**, 620-30.

Blake, D. J., Weir, A., Newey, S. E. and Davies, K. E. (2002). Function and genetics of dystrophin and dystrophin-related proteins in muscle. *Physiol Rev* **82**, 291-329.

Bodine, S. C., Latres, E., Baumhueter, S., Lai, V. K., Nunez, L., Clarke, B. A., Poueymirou, W. T., Panaro, F. J., Na, E., Dharmarajan, K. et al. (2001). Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science* **294**, 1704-8.

Brack, A. S., Conboy, I. M., Conboy, M. J., Shen, J. and Rando, T. A. (2008). A temporal switch from Notch to Wnt signaling in muscle stem cells is necessary for normal adult myogenesis. *Cell Stem Cell* **2**, 50-59.

Brack, A. S., Conboy, M. J., Roy, S., Lee, M., Kuo, C. J., Keller, C. and Rando, T. A. (2007). Increased Wnt signaling during aging alters muscle stem cell fate and increases fibrosis. *Science* **317**, 807-810.

Braun, T., Rudnicki, M. A., Arnold, H. H. and Jaenisch, R. (1992). Targeted Inactivation of the Muscle Regulatory Gene Myf-5 Results in Abnormal Rib Development and Perinatal Death. *Cell* **71**, 369-382.

Bruick, R. K. (2003). Oxygen sensing in the hypoxic response pathway: regulation of the hypoxia-inducible transcription factor. *Genes & Development* **17**, 2614-2623.

Caniggia, I., Mostachfi, H., Winter, J., Gassmann, M., Lye, S. J., Kuliszewski, M. and Post, M. (2000). Hypoxia-inducible factor-1 mediates the biological effects of oxygen on human trophoblast differentiation through TGF beta(3). *Journal of Clinical Investigation* **105**, 577-587.

Capers, C. R. (1960). Multinucleation of skeletal muscle in vitro. *J Biophys Biochem Cytol* **7**, 559-66.

Castel, D., Mourikis, P., Bartels, S. J. J., Brinkman, A. B., Tajbakhsh, S. and Stunnenberg, H. G. (2013). Dynamic binding of RBPJ is determined by Notch signaling status. *Genes & Development* **27**, 1059-1071.

Castelo-Branco, C. and Soveral, I. (2014). The immune system and aging: a review. *Gynecological Endocrinology* **30**, 16-22.

Charge, S. B. P. and Rudnicki, M. A. (2004). Cellular and molecular regulation of muscle regeneration. *Physiological Reviews* **84**, 209-238.

Chen, D., Li, Y., Zhou, Z., Wu, C., Xing, Y., Zou, X., Tian, W. and Zhang, C. (2013). HIF-1alpha inhibits Wnt signaling pathway by activating Sost expression in osteoblasts. *Plos One* **8**, e65940.

Chitnis, A. and Bally-Cuif, L. (2016). The Notch meeting: an odyssey from structure to function (vol 143, pg 547, 2016). *Development* **143**, 1229-1229.

Ciafaloni, E., Kumar, A., Liu, K., Pandya, S., Westfield, C., Fox, D. J., Caspers Conway, K. M., Cunniff, C., Mathews, K., West, N. et al. (2016). Age at onset of first signs or symptoms predicts age at loss of ambulation in Duchenne and Becker Muscular Dystrophy: Data from the MD STARnet. *J Pediatr Rehabil Med* **9**, 5-11.

Cinnamon, Y., Ben-Yair, R. and Kalcheim, C. (2006). Differential effects of N-cadherin-mediated adhesion on the development of myotomal waves. *Development* **133**, 1101-1112.

Circu, M. L. and Aw, T. Y. (2010). Reactive oxygen species, cellular redox systems, and apoptosis. *Free Radic Biol Med* **48**, 749-62.

Cisternas, P., Henriquez, J. P., Brandan, E. and Inestrosa, N. C. (2014). Wnt signaling in skeletal muscle dynamics: myogenesis, neuromuscular synapse and fibrosis. *Mol Neurobiol* **49**, 574-89.

Clanton, T. L. (2007). Hypoxia-induced reactive oxygen species formation in skeletal muscle. *Journal of Applied Physiology* **102**, 2379-2388.

Clarke, L. and van der Kooy, D. (2009). Low Oxygen Enhances Primitive and Definitive Neural Stem Cell Colony Formation by Inhibiting Distinct Cell Death Pathways. *Stem Cells* **27**, 1879-1886.

Clevers, H. and Nusse, R. (2012). Wnt/beta-catenin signaling and disease. *Cell* **149**, 1192-205.

Collins, C. A., Olsen, I., Zammit, P. S., Heslop, L., Petrie, A., Partridge, T. A. and Morgan, J. E. (2005). Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. *Cell* **122**, 289-301.

Compernolle, V., Brusselmans, K., Acker, T., Hoet, P., Tjwa, M., Beck, H., Plaisance, S., Dor, Y., Keshet, E., Lupu, F. et al. (2002). Loss of HIF-2alpha and inhibition of VEGF impair fetal lung maturation, whereas treatment with VEGF prevents fatal respiratory distress in premature mice. *Nat Med* **8**, 702-10.

Conboy, I. M. and Rando, T. A. (2002a). The regulation of Notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. *Dev Cell* **3**, 397-409.

Conboy, I. M. and Rando, T. A. (2002b). The regulation of notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. *Developmental Cell* **3**, 397-409.

Cowden Dahl, K. D., Fryer, B. H., Mack, F. A., Compernolle, V., Maltepe, E., Adelman, D. M., Carmeliet, P. and Simon, M. C. (2005). Hypoxia-inducible factors 1alpha and 2alpha regulate trophoblast differentiation. *Molecular and Cellular Biology* **25**, 10479-91.

Croker, B. A., Kiu, H. and Nicholson, S. E. (2008). SOCS regulation of the JAK/STAT signalling pathway. *Seminars in Cell & Developmental Biology* **19**, 414-22.

Davis, R. L., Weintraub, H. and Lassar, A. B. (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* **51**, 987-1000.

Day, R. M. and Suzuki, Y. J. (2005). Cell proliferation, reactive oxygen and cellular glutathione. *Dose Response* **3**, 425-42.

Dengler, V. L., Galbraith, M. D. and Espinosa, J. M. (2014). Transcriptional regulation by hypoxia inducible factors. *Critical Reviews in Biochemistry and Molecular Biology* **49**, 1-15.

Di Carlo, A., De Mori, R., Martelli, F., Pompilio, G., Capogrossi, M. C. and Germani, A. (2004). Hypoxia inhibits myogenic differentiation through accelerated MyoD degradation. *Journal of Biological Chemistry* **279**, 16332-16338.

Dubois-Ferriere, R. and Chinet, A. E. (1981). Contribution of skeletal muscle to the regulatory non-shivering thermogenesis in small mammals. *Pflugers Arch* **390**, 224-9.

Dunwoodie, S. L. (2009). The Role of Hypoxia in Development of the Mammalian Embryo. *Developmental Cell* **17**, 755-773.

Egerman, M. A. and Glass, D. J. (2014). Signaling pathways controlling skeletal muscle mass. *Crit Rev Biochem Mol Biol* **49**, 59-68.

Eliasson, P. and Jonsson, J. I. (2010). The Hematopoietic Stem Cell Niche: Low in Oxygen but a Nice Place to be. *Journal of Cellular Physiology* **222**, 17-22.

Esner, M., Meilhac, S. M., Relaix, F., Nicolas, J. F., Cossu, G. and Buckingham, M. E. (2006). Smooth muscle of the dorsal aorta shares a common clonal origin with skeletal muscle of the myotome. *Development* **133**, 737-749.

Fisher, F. M., Kleiner, S., Douris, N., Fox, E. C., Mepani, R. J., Verdeguer, F., Wu, J., Kharitonenkov, A., Flier, J. S., Maratos-Flier, E. et al. (2012). FGF21 regulates PGC-1alpha and browning of white adipose tissues in adaptive thermogenesis. *Genes Dev* **26**, 271-81.

Forristal, C. E., Wright, K. L., Hanley, N. A., Oreffo, R. O. C. and Houghton, F. D. (2010). Hypoxia inducible factors regulate pluripotency and proliferation in human embryonic stem cells cultured at reduced oxygen tensions. *Reproduction* **139**, 85-97.

Gauron, C., Rampon, C., Bouzaffour, M., Ipendey, E., Teillon, J., Volovitch, M. and Vriz, S. (2013). Sustained production of ROS triggers compensatory proliferation and is required for regeneration to proceed. *Scientific Reports* **3**.

Gehlert, S., Weber, S., Weidmann, B., Gutsche, K., Platen, P., Graf, C., Kappes-Horn, K. and Bloch, W. (2012). Cycling exercise-induced myofiber transitions in skeletal muscle depend on basal fiber type distribution. *European Journal of Applied Physiology* **112**, 2393-2402.

Gibson, M. C. and Schultz, E. (1982). The Distribution of Satellite Cells and Their Relationship to Specific Fiber Types in Soleus and Extensor Digitorum Longus Muscles. *Anatomical Record* **202**, 329-337.

Gnarra, J. R., Ward, J. M., Porter, F. D., Wagner, J. R., Devor, D. E., Grinberg, A., Emmert-Buck, M. R., Westphal, H., Klausner, R. D. and Linehan, W. M. (1997). Defective placental vasculogenesis causes embryonic lethality in VHL-deficient mice. *Proc Natl Acad Sci U S A* **94**, 9102-7.

Gomes, M. D., Lecker, S. H., Jagoe, R. T., Navon, A. and Goldberg, A. L. (2001). Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy. *Proc Natl Acad Sci U S A* **98**, 14440-5.

Gordon, W. R., Arnett, K. L. and Blacklow, S. C. (2008). The molecular logic of Notch signaling - a structural and biochemical perspective. *Journal of Cell Science* **121**, 3109-3119.

Gorlach, A. and Bonello, S. (2008). The cross-talk between NF-kappa B and HIF-1: further evidence for a significant liaison (vol 412, pg e17, 2008). *Biochemical Journal* **413**, 571-571.

Greer, S. N., Metcalf, J. L., Wang, Y. and Ohh, M. (2012). The updated biology of hypoxia-inducible factor. *Embo Journal* **31**, 2448-2460.

Gros, J., Manceau, M., Thome, V. and Marcelle, C. (2005). A common somitic origin for embryonic muscle progenitors and satellite cells. *Nature* **435**, 954-958.

Gros, J., Scaal, M. and Marcelle, C. (2004). A two-step mechanism for myotome formation in chick. *Dev Cell* **6**, 875-82.

Grounds, M. D. and Yablonka-Reuveni, Z. (1993). Molecular and cell biology of skeletal muscle regeneration. *Mol Cell Biol Hum Dis Ser* **3**, 210-56.

Gundersen, K. and Bruusgaard, J. C. (2008). Nuclear domains during muscle atrophy: nuclei lost or paradigm lost? *J Physiol* **586**, 2675-81.

Gustafsson, M. V., Zheng, X., Pereira, T., Gradin, K., Jin, S., Lundkvist, J., Ruas, J. L., Poellinger, L., Lendahl, U. and Bondesson, M. (2005a). Hypoxia requires notch signaling to maintain the undifferentiated cell state. *Dev Cell* **9**, 617-28.

Gustafsson, M. V., Zheng, X. W., Pereira, T., Gradin, K., Jin, S. B., Lundkvist, J., Ruas, J. L., Poellinger, L., Lendahl, U. and Bondesson, M. (2005b). Hypoxia requires Notch signaling to maintain the undifferentiated cell state. *Developmental Cell* **9**, 617-628.

Haghighat, A., Mader, S., Pause, A. and Sonenberg, N. (1995). Repression of cap-dependent translation by 4E-binding protein 1: competition with p220 for binding to eukaryotic initiation factor-4E. *EMBO J* **14**, 5701-9.

Halevy, O., Piestun, Y., Allouh, M. Z., Rosser, B. W., Rinkevich, Y., Reshef, R., Rozenboim, I., Wleklinski-Lee, M. and Yablonka-Reuveni, Z. (2004). Pattern of Pax7 expression during myogenesis in the posthatch chicken establishes a model for satellite cell differentiation and renewal. *Dev Dyn* **231**, 489-502.

Hall, Z. W. and Ralston, E. (1989). Nuclear Domains in Muscle-Cells. *Cell* **59**, 771-772.

Hansen, S., Baptiste, K. E., Fjeldborg, J. and Horohov, D. W. (2015). A review of the equine age-related changes in the immune system: Comparisons between human and equine aging, with focus on lung-specific immune-aging. *Ageing Research Reviews* **20**, 11-23.

Hanssen, M. J., Broeders, E., Samms, R. J., Vosselman, M. J., van der Lans, A. A., Cheng, C. C., Adams, A. C., van Marken Lichtenbelt, W. D. and Schrauwen, P. (2015). Serum FGF21 levels are associated with brown adipose tissue activity in humans. *Sci Rep* **5**, 10275.

Hara, K., Yonezawa, K., Kozlowski, M. T., Sugimoto, T., Andrabi, K., Weng, Q. P., Kasuga, M., Nishimoto, I. and Avruch, J. (1997). Regulation of eIF-4E BP1 phosphorylation by mTOR. *Journal of Biological Chemistry* **272**, 26457-63.

Hartman, M. A. and Spudich, J. A. (2012). The myosin superfamily at a glance. *Journal of Cell Science* **125**, 1627-1632.

Hasty, P., Bradley, A., Morris, J. H., Edmondson, D. G., Venuti, J. M., Olson, E. N. and Klein, W. H. (1993). Muscle Deficiency and Neonatal Death in Mice with a Targeted Mutation in the Myogenin Gene. *Nature* **364**, 501-506.

Hawke, T. J. and Garry, D. J. (2001). Myogenic satellite cells: physiology to molecular biology. *J Appl Physiol (1985)* **91**, 534-51.

Hayward, P., Kalmar, T. and Arias, A. M. (2008). Wnt/Notch signalling and information processing during development. *Development* **135**, 411-424.

Heredia, J. E., Mukundan, L., Chen, F. M., Mueller, A. A., Deo, R. C., Locksley, R. M., Rando, T. A. and Chawla, A. (2013). Type 2 innate signals stimulate fibro/adipogenic progenitors to facilitate muscle regeneration. *Cell* **153**, 376-88.

Higashifujime, S. (1981). Active Movement of Bundles of Actin and Myosin-Filaments from Muscle - a Simple-Model for Cell Motility. *Cold Spring Harbor Symposia on Quantitative Biology* **46**, 69-75.

Ho, H. H. and Ivashkiv, L. B. (2006). Role of STAT3 in type I interferon responses - Negative regulation of STAT1-dependent inflammatory gene activation. *Journal of Biological Chemistry* **281**, 14111-14118.

Hofmann, T., Elbelt, U. and Stengel, A. (2014). Irisin as a muscle-derived hormone stimulating thermogenesis--a critical update. *Peptides* **54**, 89-100.

Hondares, E., Rosell, M., Gonzalez, F. J., Giralt, M., Iglesias, R. and Villarroya, F. (2010). Hepatic FGF21 Expression Is Induced at Birth via PPAR alpha in Response to Milk Intake and Contributes to Thermogenic Activation of Neonatal Brown Fat. *Cell Metabolism* **11**, 206-212.

Honma, S., Kawamoto, T., Takagi, Y., Fujimoto, K., Sato, F., Noshiro, M., Kato, Y. and Honma, K. (2002). Dec1 and Dec2 are regulators of the mammalian molecular clock. *Nature* **419**, 841-844.

Howald, H. and Hoppeler, H. (2003). Performing at extreme altitude: muscle cellular and subcellular adaptations. *European Journal of Applied Physiology* **90**, 360-364.

Howard, J. (1997). Molecular motors: structural adaptations to cellular functions. *Nature* **389**, 561-567.

Hutcheson, D. A., Zhao, J., Merrell, A., Haldar, M. and Kardon, G. (2009). Embryonic and fetal limb myogenic cells are derived from developmentally distinct progenitors and have different requirements for beta-catenin. *Genes & Development* **23**, 997-1013.

Iso, T., Kedes, L. and Hamamori, Y. (2003). HES and HERP families: Multiple effectors of the Notch signaling pathway. *Journal of Cellular Physiology* **194**, 237-255.

Ivan, M., Kondo, K., Yang, H. F., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S. and Kaelin, W. G. (2001). HIF alpha targeted for VHL-mediated destruction by proline hydroxylation: Implications for O-2 sensing. *Science* **292**, 464-468.

Ivashkiv, L. B. and Donlin, L. T. (2014). Regulation of type I interferon responses. *Nature Reviews Immunology* **14**, 36-49.

Iyer, N. V., Kotch, L. E., Agani, F., Leung, S. W., Laughner, E., Wenger, R. H., Gassmann, M., Gearhart, J. D., Lawler, A. M., Yu, A. Y. et al. (1998). Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. *Genes Dev* **12**, 149-62.

Jaakkola, P., Mole, D. R., Tian, Y. M., Wilson, M. I., Gielbert, J., Gaskell, S. J., von Kriegsheim, A., Hebestreit, H. F., Mukherji, M., Schofield, C. J. et al. (2001). Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O-2-regulated prolyl hydroxylation. *Science* **292**, 468-472.

Jarecki, J., Johnson, E. and Krasnow, M. A. (1999). Oxygen regulation of airway branching in Drosophila is mediated by branchless FGF. *Cell* **99**, 211-220.

Joe, A. W. B., Yi, L., Natarajan, A., Le Grand, F., So, L., Wang, J., Rudnicki, M. A. and Rossi, F. M. V. (2010). Muscle injury activates resident fibro/adipogenic progenitors that facilitate myogenesis. *Nature Cell Biology* **12**, 153-U144.

Jones, A. E., Price, F. D., Le Grand, F., Soleimani, V. D., Dick, S. A., Megeney, L. A. and Rudnicki, M. A. (2015). Wnt/beta-catenin controls follistatin signalling to regulate satellite cell myogenic potential. *Skelet Muscle* **5**, 14.

Kablar, B., Asakura, A., Krastel, K., Ying, C. Y., May, L. L., Goldhamer, D. J. and Rudnicki, M. A. (1998). MyoD and Myf-5 define the specification of musculature of distinct embryonic origin. *Biochemistry and Cell Biology-Biochimie Et Biologie Cellulaire* **76**, 1079-1091.

Kaidi, A., Williams, A. C. and Paraskeva, C. (2007). Interaction between beta-catenin and HIF-1 promotes cellular adaptation to hypoxia. *Nature Cell Biology* **9**, 210-U113.

Kalcheim, C. and Ben-Yair, R. (2005). Cell rearrangements during development of the somite and its derivatives. *Curr Opin Genet Dev* **15**, 371-80.

Kambadur, R., Sharma, M., Smith, T. P. and Bass, J. J. (1997). Mutations in myostatin (GDF8) in double-muscled Belgian Blue and Piedmontese cattle. *Genome Res* **7**, 910-6.

Kanning, K. C., Kaplan, A. and Henderson, C. E. (2010). Motor Neuron Diversity in Development and Disease. *Annual Review of Neuroscience, Vol 33* **33**, 409-440.

Kassar-Duchossoy, L., Giacone, E., Gayraud-Morel, B., Jory, A., Gomes, D. and Tajbakhsh, S. (2005). Pax3/Pax7 mark a novel population of primitive myogenic cells during development. *Genes & Development* **19**, 1426-1431.

Katz, B. and Miledi, R. (1961). The localized action of end-plate drugs' in the twitch fibres of the frog. *J Physiol* **155**, 399-415.

Kaufman, D. S. (2010). HIF hits Wnt in the stem cell niche. *Nature Cell Biology* **12**, 926-927.

Kemaladewi, D. U., de Gorter, D. J., Aartsma-Rus, A., van Ommen, G. J., ten Dijke, P., t Hoen, P. A. and Hoogaars, W. M. (2012). Cell-type specific regulation of myostatin signaling. *Faseb Journal* **26**, 1462-72.

Kim, C. H., Neiswender, H., Baik, E. J., Xiong, W. C. and Mei, L. (2008). beta-catenin interacts with MyoD and regulates its transcription activity. *Molecular and Cellular Biology* **28**, 2941-2951.

Kingham, P. J. and Pocock, J. M. (2001). Microglial secreted cathepsin B induces neuronal apoptosis. *Journal of Neurochemistry* **76**, 1475-1484.

Klaus, A. and Birchmeier, W. (2008). Wnt signalling and its impact on development and cancer. *Nat Rev Cancer* **8**, 387-98.

Kopan, R., Nye, J. S. and Weintraub, H. (1994). The Intracellular Domain of Mouse Notch - a Constitutively Activated Repressor of Myogenesis Directed at the Basic Helix-Loop-Helix Region of Myod. *Development* **120**, 2385-2396.

Kozak, K. R., Abbott, B. and Hankinson, O. (1997). ARNT-deficient mice and placental differentiation. *Developmental Biology* **191**, 297-305.

Kuang, S., Charge, S. B., Seale, P., Huh, M. and Rudnicki, M. A. (2006). Distinct roles for Pax7 and Pax3 in adult regenerative myogenesis. *Journal of Cell Biology* **172**, 103-113.

Kuang, S., Gillespie, M. A. and Rudnicki, M. A. (2008). Niche regulation of muscle satellite cell self-renewal and differentiation. *Cell Stem Cell* **2**, 22-31.

Kuang, S. and Rudnicki, M. A. (2008). The emerging biology of satellite cells and their therapeutic potential. *Trends Mol Med* **14**, 82-91.

Kuang, S. H., Kuroda, K., Le Grand, F. and Rudnicki, M. A. (2007). Asymmetric self-renewal and commitment of satellite stem cells in muscle. *Cell* **129**, 999-1010.

Kwon, C., Cheng, P., King, I. N., Andersen, P., Shenje, L., Nigam, V. and Srivastava, D. (2011). Notch post-translationally regulates beta-catenin protein in stem and progenitor cells. *Nature Cell Biology* **13**, 1244-U169.

Lasky, J. L. and Wu, H. (2005). Notch signaling, brain development, and human disease. *Pediatric Research* **57**, 104r-109r.

Lecomte, V., Meugnier, E., Euthine, V., Durand, C., Freyssenet, D., Nemoz, G., Rome, S., Vidal, H. and Lefai, E. (2010). A New Role for Sterol Regulatory Element Binding Protein 1 Transcription Factors in the Regulation of Muscle Mass and Muscle Cell Differentiation. *Molecular and Cellular Biology* **30**, 1182-1198.

Lehar, S. M., Dooley, J., Farr, A. G. and Bevan, M. J. (2005). Notch ligands delta1 and jagged1 transmit distinct signals to T-cell precursors. *Blood* **105**, 1440-1447.

Lemos, D. R., Babaeijandaghi, F., Low, M., Chang, C. K., Lee, S. T., Fiore, D., Zhang, R. H., Natarajan, A., Nedospasov, S. A. and Rossi, F. M. V. (2015). Nilotinib reduces muscle fibrosis in chronic muscle injury by promoting TNF-mediated apoptosis of fibro/adipogenic progenitors. *Nature Medicine* **21**, 786-+.

Li, Q. F., Wang, X. R., Yang, Y. W. and Lin, H. (2006). Hypoxia upregulates hypoxia inducible factor (HIF)-3 alpha expression in lung epithelial cells: characterization and comparison with HIF-1 alpha. *Cell Research* **16**, 548-558.

Li, Y. X., Zhang, H., Xie, M. X., Hu, M. W., Ge, S. J., Yang, D. F., Wan, Y. S. and Yan, B. F. (2002). Abundant expression of Dec1/stra13/sharp2 in colon carcinoma: its antagonizing role in serum deprivation-induced apoptosis and selective inhibition of procaspase activation. *Biochemical Journal* **367**, 413-422.

Liu, D., Black, B. L. and Derynck, R. (2001). TGF-beta inhibits muscle differentiation through functional repression of myogenic transcription factors by Smad3. *Genes & Development* **15**, 2950-2966.

Liu, W. Y., Liu, Y. Q., Lai, X. S. and Kuang, S. H. (2012a). Intramuscular adipose is derived from a non-Pax3 lineage and required for efficient regeneration of skeletal muscles. *Developmental Biology* **361**, 27-38.

Liu, W. Y., Wen, Y. F., Bi, P. P., Lai, X. S., Liu, X. S., Liu, X. Q. and Kuang, S. H. (2012b). Hypoxia promotes satellite cell self-renewal and enhances the efficiency of myoblast transplantation. *Development* **139**, 2857-2865.

Liu, Y., Wang, L., Lin, X. Y., Wang, J., Yu, J. H., Miao, Y. and Wang, E. H. (2013). The transcription factor DEC1 (BHLHE40/STRA13/SHARP-2) is negatively associated with TNM stage in non-small-cell lung cancer and inhibits the proliferation through cyclin D1 in A549 and BE1 cells. *Tumor Biology* **34**, 1641-1650.

Luo, D., Renault, V. M. and Rando, T. A. (2005). The regulation of Notch signaling in muscle stem cell activation and postnatal myogenesis. *Seminars in Cell & Developmental Biology* **16**, 612-622.

MacDonald, B. T., Tamai, K. and He, X. (2009). Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev Cell* **17**, 9-26.

Mahon, P. C., Hirota, K. and Semenza, G. L. (2001). FIH-1: a novel protein that interacts with HIF-1 alpha and VHL to mediate repression of HIF-1 transcriptional activity. *Genes & Development* **15**, 2675-2686.

Majmundar, A. J., Lee, D. S. M., Skuli, N., Mesquita, R. C., Kim, M. N., Yodh, A. G., Nguyen-McCarty, M., Li, B. and Simon, M. C. (2015). HIF modulation of Wnt signaling regulates skeletal myogenesis in vivo. *Development* **142**, 2405-+.

Majmundar, A. J., Skuli, N., Mesquita, R. C., Kim, M. N., Yodh, A. G., Nguyen-McCarty, M. and Simon, M. C. (2012). O-2 Regulates Skeletal Muscle Progenitor Differentiation through Phosphatidylinositol 3-Kinase/AKT Signaling. *Molecular and Cellular Biology* **32**, 36-49.

Mammucari, C., Milan, G., Romanello, V., Masiero, E., Rudolf, R., Del Piccolo, P., Burden, S. J., Di Lisi, R., Sandri, C., Zhao, J. et al. (2007). FoxO3 controls autophagy in skeletal muscle in vivo. *Cell Metab* **6**, 458-71.

Mason, S. D., Howlett, R. A., Kim, M. J., Olfert, I. M., Hogan, M. C., McNulty, W., Hickey, R. P., Wagner, P. D., Kahn, C. R., Giordano, F. J. et al. (2004). Loss of skeletal muscle HIF-1 alpha results in altered exercise endurance. *Plos Biology* **2**, 1540-1548.

Massague, J. (2012). TGFbeta signalling in context. *Nat Rev Mol Cell Biol* **13**, 616-30.

Matsuura, H., Ichiki, T., Ikeda, J., Takeda, K., Miyazaki, R., Hashimoto, T., Narabayashi, E., Kitamoto, S., Tokunou, T. and Sunagawa, K. (2011). Inhibition of prolyl hydroxylase domain-containing protein downregulates vascular angiotensin II type 1 receptor. *Hypertension* **58**, 386-93.

Mauro, A. (1961). Satellite cell of skeletal muscle fibers. *J Biophys Biochem Cytol* **9**, 493-5.

Maxwell, P. H., Wiesener, M. S., Chang, G. W., Clifford, S. C., Vaux, E. C., Cockman, M. E., Wykoff, C. C., Pugh, C. W., Maher, E. R. and Ratcliffe, P. J. (1999). The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature* **399**, 271-275.

Mazumdar, J., O'Brien, W. T., Johnson, R. S., LaManna, J. C., Chavez, J. C., Klein, P. S. and Simon, M. C. (2010). O-2 regulates stem cells through Wnt/beta-catenin signalling. *Nature Cell Biology* **12**, 1007-1013.

McMahon, S., Charbonneau, M., Grandmont, S., Richard, D. E. and Dubois, C. M. (2006). Transforming growth factor beta 1 induces hypoxia-inducible factor-1 stabilization through selective inhibition of PHD2 expression. *Journal of Biological Chemistry* **281**, 24171-24181.

McPherron, A. C., Lawler, A. M. and Lee, S. J. (1997). Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature* **387**, 83-90.

Merrick, M. A. (2002). Secondary injury after musculoskeletal trauma: a review and update. *J Athl Train* **37**, 209-17.

Miscia, S., Marchisio, M., Grilli, A., Di Valerio, V., Centurione, L., Sabatino, G., Garaci, F., Zauli, G., Bonvini, E. and Di Baldassarre, A. (2002). Tumor necrosis factor alpha (TNF-alpha) activates Jak1/Stat3-Stat5B signaling through IR-1 in human B cells. *Cell Growth & Differentiation* **13**, 13-18.

Mitch, W. E. and Goldberg, A. L. (1996). Mechanisms of muscle wasting. The role of the ubiquitin-proteasome pathway. *N Engl J Med* **335**, 1897-905.

Montero, J., Dutta, C., van Bodegom, D., Weinstock, D. and Letai, A. (2013). p53 regulates a non-apoptotic death induced by ROS. *Cell Death and Differentiation* **20**, 1465-74.

Moon, H. Y., Becke, A., Berron, D., Becker, B., Sah, N., Benoni, G., Janke, E., Lubejko, S. T., Greig, N. H., Mattison, J. A. et al. (2016). Running-Induced Systemic Cathepsin B Secretion Is Associated with Memory Function. *Cell Metab*.

Morgan, M. J. and Liu, Z. G. (2011). Crosstalk of reactive oxygen species and NF-kappaB signaling. *Cell Res* **21**, 103-15.

Morriss, G. M. and New, D. A. T. (1979). Effect of Oxygen Concentration on Morphogenesis of Cranial Neural Folds and Neural Crest in Cultured Rat Embryos. *Journal of Embryology and Experimental Morphology* **54**, 17-35.

Mukherjee, T., Kim, W. S., Mandal, L. and Banerjee, U. (2011). Interaction Between Notch and Hif-alpha in Development and Survival of Drosophila Blood Cells. *Science* **332**, 1210-1213.

Nabeshima, Y., Hanaoka, K., Hayasaka, M., Esumi, E., Li, S. W., Nonaka, I. and Nabeshima, Y. (1993). Myogenin Gene Disruption Results in Perinatal Lethality Because of Severe Muscle Defect. *Nature* **364**, 532-535.

Naito, A. T., Sumida, T., Nomura, S., Liu, M. L., Higo, T., Nakagawa, A., Okada, K., Sakai, T., Hashimoto, A., Hara, Y. et al. (2012). Complement C1q activates canonical Wnt signaling and promotes aging-related phenotypes. *Cell* **149**, 1298-313.

Nowak, K. J. and Davies, K. E. (2004). Duchenne muscular dystrophy and dystrophin: pathogenesis and opportunities for treatment. *EMBO Rep* **5**, 872-6.

O'Shea, J. J., Schwartz, D. M., Villarino, A. V., Gadina, M., McInnes, I. B. and Laurence, A. (2015). The JAK-STAT pathway: impact on human disease and therapeutic intervention. *Annu Rev Med* **66**, 311-28.

Olguin, H. C. and Olwin, B. B. (2004). Pax-7 up-regulation inhibits myogenesis and cell cycle progression in satellite cells: a potential mechanism for self-renewal. *Developmental Biology* **275**, 375-388.

Otto, A., Schmidt, C., Luke, G., Allen, S., Valasek, P., Muntoni, F., Lawrence-Watt, D. and Patel, K. (2008a). Canonical Wnt signalling induces satellite-cell proliferation during adult skeletal muscle regeneration. *Journal of Cell Science* **121**, 2939-2950.

Otto, A., Schmidt, C., Luke, G., Allen, S., Valasek, P., Muntoni, F., Lawrence-Watt, D. and Patel, K. (2008b). Canonical Wnt signalling induces satellite-cell proliferation during adult skeletal muscle regeneration. *Journal of Cell Science* **121**, 2939-50.

Pannerec, A., Formicola, L., Besson, V., Marazzi, G. and Sassoon, D. A. (2013). Defining skeletal muscle resident progenitors and their cell fate potentials. *Development* **140**, 2879-2891.

Pavlath, G. K., Rich, K., Webster, S. G. and Blau, H. M. (1989). Localization of Muscle Gene-Products in Nuclear Domains. *Nature* **337**, 570-573.

Payne, A. M., Zheng, Z., Gonzalez, E., Wang, Z. M., Messi, M. L. and Delbono, O. (2004). External Ca(2+)-dependent excitation--contraction coupling in a population of ageing mouse skeletal muscle fibres. *J Physiol* **560**, 137-55.

Perez-Ruiz, A., Ono, Y., Gnocchi, V. F. and Zammit, P. S. (2008). beta-Catenin promotes self-renewal of skeletal-muscle satellite cells. *Journal of Cell Science* **121**, 1373-82.

Pisani, D. F. and Dechesne, C. A. (2005). Skeletal muscle HIF-1 alpha expression is dependent on muscle fiber type. *Journal of General Physiology* **126**, 173-178.

Pistollato, F., Chen, H. L., Rood, B. R., Zhang, H. Z., D'Avella, D., Denaro, L., Gardiman, M., te Kronnie, G., Schwartz, P. H., Favaro, E. et al. (2009). Hypoxia and HIF1alpha repress the differentiative effects of BMPs in high-grade glioma. *Stem Cells* **27**, 7-17.

Pistollato, F., Rampazzo, E., Persano, L., Abbadi, S., Frasson, C., Denaro, L., D'Avella, D., Panchision, D. M., Della Puppa, A., Scienza, R. et al. (2010). Interaction of hypoxia-inducible factor-1alpha and Notch signaling regulates medulloblastoma precursor proliferation and fate. *Stem Cells* **28**, 1918-29.

Pownall, M. E., Gustafsson, M. K. and Emerson, C. P. (2002). Myogenic regulatory factors and the specification of muscle progenitors in vertebrate embryos. *Annual Review of Cell and Developmental Biology* **18**, 747-783.

Price, F. D., von Maltzahn, J., Bentzinger, C. F., Dumont, N. A., Yin, H., Chang, N. C., Wilson, D. H., Frenette, J. and Rudnicki, M. A. (2015). Inhibition of JAK-STAT signaling stimulates adult satellite cell function (vol 20, pg 1174, 2014). *Nature Medicine* **21**, 414-414.

Qing, Y. L. and Stark, G. R. (2004). Alternative activation of STAT1 and STAT3 in response to interferon-gamma. *Journal of Biological Chemistry* **279**, 41679-41685.

Raggatt, L. J. and Partridge, N. C. (2010). Cellular and molecular mechanisms of bone remodeling. *Journal of Biological Chemistry* **285**, 25103-8.

Rajpurohit, R., Koch, C. J., Tao, Z. L., Teixeira, C. M. and Shapiro, I. M. (1996). Adaptation of chondrocytes to low oxygen tension: Relationship between hypoxia and cellular metabolism. *Journal of Cellular Physiology* **168**, 424-432.

Rathbone, C. R., Yamanouchi, K., Chen, X. K., Nevoret-Bell, C. J., Rhoads, R. P. and Allen, R. E. (2011). Effects of transforming growth factor-beta (TGF-beta1) on satellite cell activation and survival during oxidative stress. *J Muscle Res Cell Motil* **32**, 99-109.

Rawlings, J. S., Rosler, K. M. and Harrison, D. A. (2004). The JAK/STAT signaling pathway. *Journal of Cell Science* **117**, 1281-1283.

Ream, M., Ray, A. M., Chandra, R. and Chikaraishi, D. M. (2008). Early fetal hypoxia leads to growth restriction and myocardial thinning. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology* **295**, R583-R595.

Relaix, F., Rocancourt, D., Mansouri, A. and Buckingham, M. (2004). Divergent functions of murine Pax3 and Pax7 in limb muscle development. *Genes Dev* **18**, 1088-105.

Relaix, F., Rocancourt, D., Mansouri, A. and Buckingham, M. (2005). A Pax3/Pax7-dependent population of skeletal muscle progenitor cells. *Nature* **435**, 948-953.

Relaix, F. and Zammit, P. S. (2012). Satellite cells are essential for skeletal muscle regeneration: the cell on the edge returns centre stage. *Development* **139**, 2845-2856.

Rowland, L. A., Bal, N. C. and Periasamy, M. (2015). The role of skeletal-muscle-based thermogenic mechanisms in vertebrate endothermy. *Biol Rev Camb Philos Soc* **90**, 1279-97.

Rudnicki, M. A., Schnegelsberg, P. N. J., Stead, R. H., Braun, T., Arnold, H. H. and Jaenisch, R. (1993). Myod or Myf-5 Is Required for the Formation of Skeletal-Muscle. *Cell* **75**, 1351-1359.

Sabharwal, S. S. and Schumacker, P. T. (2014). Mitochondrial ROS in cancer: initiators, amplifiers or an Achilles' heel? *Nature Reviews Cancer* **14**, 709-721.

Sabourin, L. A. and Rudnicki, M. A. (2000). The molecular regulation of myogenesis. *Clinical Genetics* **57**, 16-25.

Sacco, A., Doyonnas, R., Kraft, P., Vitorovic, S. and Blau, H. M. (2008). Self-renewal and expansion of single transplanted muscle stem cells. *Nature* **456**, 502-506.

Saini, J., McPhee, J. S., Al-Dabbagh, S., Stewart, C. E. and Al-Shanti, N. (2016). Regenerative function of immune system: Modulation of muscle stem cells. *Ageing Res Rev* **27**, 67-76.

Sartori, R., Gregorevic, P. and Sandri, M. (2014). TGF beta and BMP signaling in skeletal muscle: potential significance for muscle-related disease. *Trends in Endocrinology and Metabolism* **25**, 464-471.

Sartori, R., Schirwis, E., Blaauw, B., Bortolanza, S., Zhao, J. H., Enzo, E., Stantzou, A., Mouisel, E., Toniolo, L., Ferry, A. et al. (2013). BMP signaling controls muscle mass. *Nature Genetics* **45**, 1309-+.

Schiaffino, S. and Reggiani, C. (2011). Fiber types in mammalian skeletal muscles. *Physiol Rev* **91**, 1447-531.

Schienda, J., Engleka, K. A., Jun, S., Hansen, M. S., Epstein, J. A., Tabin, C. J., Kunkel, L. M. and Kardon, G. (2006). Somitic origin of limb muscle satellite and side population cells. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 945-950.

Schultz, E., Gibson, M. C. and Champion, T. (1978). Satellite cells are mitotically quiescent in mature mouse muscle: an EM and radioautographic study. *J Exp Zool* **206**, 451-6.

Schulz-Schaeffer, W. J. (2016). Camptocormia in Parkinson's Disease: A Muscle Disease Due to Dysregulated Proprioceptive Polysynaptic Reflex Arch. *Frontiers in Aging Neuroscience* **8**, 128.

Seale, P., Sabourin, L. A., Girgis-Gabardo, A., Mansouri, A., Gruss, P. and Rudnicki, M. A. (2000). Pax7 is required for the specification of myogenic satellite cells. *Cell* **102**, 777-786.

Semenza, G. L. (2003). Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* **3**, 721-32.

Semenza, G. L. (2012). Hypoxia-Inducible Factors in Physiology and Medicine. *Cell* **148**, 399-408.

Semenza, G. L. (2014). Oxygen Sensing, Hypoxia-Inducible Factors, and Disease Pathophysiology. *Annual Review of Pathology: Mechanisms of Disease, Vol 9* **9**, 47-71.

Semenza, G. L. and Wang, G. L. (1992). A Nuclear Factor Induced by Hypoxia Via Denovo Protein-Synthesis Binds to the Human Erythropoietin Gene Enhancer at a Site Required for Transcriptional Activation. *Molecular and Cellular Biology* **12**, 5447-5454.

Shi, Y. and Massague, J. (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* **113**, 685-700.

Shweiki, D., Itin, A., Soffer, D. and Keshet, E. (1992). Vascular Endothelial Growth-Factor Induced by Hypoxia May Mediate Hypoxia-Initiated Angiogenesis. *Nature* **359**, 843-845.

Simon, M. C. (2008). The role of oxygen availability in embryonic development and stem cell function. *Birth Defects Research Part a-Clinical and Molecular Teratology* **82**, 314-314.

Simon, M. C. and Keith, B. (2008). The role of oxygen availability in embryonic development and stem cell function. *Nature Reviews Molecular Cell Biology* **9**, 285-296.

Simon, M. C., Ramirez-Bergeron, D., Mack, F., Hu, C. J., Pan, Y. and Mansfield, K. (2002). Hypoxia, HIFs, and cardiovascular development. *Cold Spring Harb Symp Quant Biol* **67**, 127-32.

Snow, M. H. (1983). A Quantitative Ultrastructural Analysis of Satellite Cells in Denervated Fast and Slow Muscles of the Mouse. *Anatomical Record* **207**, 593-604.

Son, Y., Kim, S., Chung, H. T. and Pae, H. O. (2013). Reactive oxygen species in the activation of MAP kinases. *Methods Enzymol* **528**, 27-48.

Spiegelman, B. M. (1998). PPAR-gamma: adipogenic regulator and thiazolidinedione receptor. *Diabetes* **47**, 507-14.

Sun, H., Li, L., Vercherat, C., Gulbagci, N. T., Acharjee, S., Li, J. L., Chung, T. K., Thin, T. H. and Taneja, R. (2007). Stra13 regulates satellite cell activation by antagonizing Notch signaling. *Journal of Cell Biology* **177**, 647-657.

Suzuki, A., Pelikan, R. C. and Iwata, J. (2015). WNT/beta-Catenin Signaling Regulates Multiple Steps of Myogenesis by Regulating Step-Specific Targets. *Molecular and Cellular Biology* **35**, 1763-1776.

Tanaka, S., Terada, K. and Nohno, T. (2011). Canonical Wnt signaling is involved in switching from cell proliferation to myogenic differentiation of mouse myoblast cells. *J Mol Signal* **6**, 12.

Tapscott, S. J., Davis, R. L., Thayer, M. J., Cheng, P. F., Weintraub, H. and Lassar, A. B. (1988). MyoD1: a nuclear phosphoprotein requiring a Myc homology region to convert fibroblasts to myoblasts. *Science* **242**, 405-11.

Tian, H., Biehs, B., Chiu, C., Siebel, C. W., Wu, Y., Costa, M., de Sauvage, F. J. and Klein, O. D. (2015). Opposing Activities of Notch and Wnt Signaling Regulate Intestinal Stem Cells and Gut Homeostasis. *Cell Reports* **11**, 33-42.

Tidball, J. G. and Villalta, S. A. (2010). Regulatory interactions between muscle and the immune system during muscle regeneration. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology* **298**, R1173-R1187.

Tierney, M. T., Aydogdu, T., Sala, D., Malecova, B., Gatto, S., Puri, P. L., Latella, L. and Sacco, A. (2014). STAT3 signaling controls satellite cell expansion and skeletal muscle repair. *Nat Med* **20**, 1182-6.

Toth, K. G., Mckay, B. R., De Lisio, M., Little, J. P., Tarnopolsky, M. A. and Parise, G. (2011). IL-6 Induced STAT3 Signalling Is Associated with the Proliferation of Human Muscle Satellite Cells Following Acute Muscle Damage. *Plos One* **6**.

Trensz, F., Haroun, S., Cloutier, A., Richter, M. V. and Grenier, G. (2010). A muscle resident cell population promotes fibrosis in hindlimb skeletal muscles of mdx mice through the Wnt canonical pathway. *American Journal of Physiology-Cell Physiology* **299**, C939-C947.

Tseng, W. P., Yang, S. N., Lai, C. H. and Tang, C. H. (2010). Hypoxia induces BMP-2 expression via ILK, Akt, mTOR, and HIF-1 pathways in osteoblasts. *Journal of Cellular Physiology* **223**, 810-8.

Turner, C. and Hilton-Jones, D. (2010). The myotonic dystrophies: diagnosis and management. *Journal of Neurology Neurosurgery and Psychiatry* **81**, 358-367.

Uezumi, A., Fukada, S., Yamada, H., Takeda, S. and Tsuchida, K. (2010). Mesenchymal progenitors distinct from muscle satellite cells contribute to ectopic fat cell formation in skeletal muscle. *Differentiation* **80**, S41-S41.

Urciuolo, A., Quarta, M., Morbidoni, V., Gattazzo, F., Molon, S., Grumati, P., Montemurro, F., Tedesco, F. S., Blaauw, B., Cossu, G. et al. (2013). Collagen VI regulates satellite cell self-renewal and muscle regeneration. *Nat Commun* **4**, 1964.

van Uden, P., Kenneth, N. S. and Rocha, S. (2008). Regulation of hypoxia-inducible factor-1 alpha by NF-kappa B. *Biochemical Journal* **412**, 477-484.

Vasyutina, E., Lenhard, D. C., Wende, H., Erdmann, B., Epstein, J. A. and Birchmeier, C. (2007). RBP-J (Rbpsuh) is essential to maintain muscle progenitor cells and to generate satellite cells. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 4443-4448.

Verbon, E. H., Post, J. A. and Boonstra, J. (2012). The influence of reactive oxygen species on cell cycle progression in mammalian cells. *Gene* **511**, 1-6.

Villa, J. C., Chiu, D., Brandes, A. H., Escorcia, F. E., Villa, C. H., Maguire, W. F., Hu, C. J., de Stanchina, E., Simon, M. C., Sisodia, S. S. et al. (2014a). Nontranscriptional Role of Hif-1 alpha in Activation of gamma-Secretase and Notch Signaling in Breast Cancer. *Cell Reports* **8**, 1077-1092.

Villa, J. C., Chiu, D., Brandes, A. H., Escorcia, F. E., Villa, C. H., Maguire, W. F., Hu, C. J., de Stanchina, E., Simon, M. C., Sisodia, S. S. et al. (2014b). Nontranscriptional role of Hif-1alpha in activation of gamma-secretase and notch signaling in breast cancer. *Cell Reports* **8**, 1077-92.

Wang, C., Liu, W. Y., Liu, Z. J., Chen, L., Liu, X. Q. and Kuang, S. H. (2015). Hypoxia Inhibits Myogenic Differentiation through p53 Protein-dependent Induction of Bhlhe40 Protein. *Journal of Biological Chemistry* **290**, 29707-29716.

Wang, G. L., Jiang, B. H., Rue, E. A. and Semenza, G. L. (1995). Hypoxia-Inducible Factor-1 Is a Basic-Helix-Loop-Helix-Pas Heterodimer Regulated by Cellular O-2 Tension. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 5510-5514.

Wang, W., Kissig, M., Rajakumari, S., Huang, L., Lim, H. W., Won, K. J. and Seale, P. (2014a). Ebf2 is a selective marker of brown and beige adipogenic precursor cells. *Proc Natl Acad Sci U S A* **111**, 14466-71.

Wang, Y. X., Dumont, N. A. and Rudnicki, M. A. (2014b). Muscle stem cells at a glance. *Journal of Cell Science* **127**, 4543-4548.

Watanabe, A., Ichiki, T., Sankoda, C., Takahara, Y., Ikeda, J., Inoue, E., Tokunou, T., Kitamoto, S. and Sunagawa, K. (2014). Suppression of abdominal aortic aneurysm formation by inhibition of prolyl hydroxylase domain protein through attenuation of inflammation and extracellular matrix disruption. *Clinical Science* **126**, 671-678.

Weidemann, A. and Johnson, R. S. (2008). Biology of HIF-1 alpha. *Cell Death and Differentiation* **15**, 621-627.

Weir, L., Robertson, D., Leigh, I. M., Vass, J. K. and Panteleyev, A. A. (2011). Hypoxia-mediated control of HIF/ARNT machinery in epidermal keratinocytes. *Biochimica Et Biophysica Acta-Molecular Cell Research* **1813**, 60-72.

Yablonka-Reuveni, Z., Danoviz, M. E., Phelps, M. and Stuelsatz, P. (2015). Myogenic-specific ablation of Fgfr1 impairs FGF2-mediated proliferation of satellite cells at the myofiber niche but does not abolish the capacity for muscle regeneration. *Frontiers in Aging Neuroscience* **7**.

Yablonka-Reuveni, Z. and Rivera, A. J. (1994). Temporal expression of regulatory and structural muscle proteins during myogenesis of satellite cells on isolated adult rat fibers. *Dev Biol* **164**, 588-603.

Yoshida, T., Hashimura, M., Mastumoto, T., Tazo, Y., Inoue, H., Kuwata, T. and Saegusa, M. (2013). Transcriptional upregulation of HIF-1 alpha by NF-kappa B/p65 and its associations with beta-catenin/p300 complexes in endometrial carcinoma cells. *Laboratory Investigation* **93**, 1184-1193.

You, J. S., Anderson, G. B., Dooley, M. S. and Hornberger, T. A. (2015). The role of mTOR signaling in the regulation of protein synthesis and muscle mass during immobilization in mice. *Dis Model Mech* **8**, 1059-69.

Yuan, X., Wu, H., Xu, H. X., Xiong, H. H., Chu, Q., Yu, S. Y., Wu, G. S. and Wu, K. M. (2015). Notch signaling: An emerging therapeutic target for cancer treatment. *Cancer Letters* **369**, 20-27.

Yun, Z., Lin, Q. and Giaccia, A. J. (2005). Adaptive myogenesis under hypoxia. *Molecular and Cellular Biology* **25**, 3040-55.

Yun, Z., Maecker, H. L., Johnson, R. S. and Giaccia, A. J. (2002). Inhibition of PPAR gamma 2 gene expression by the HIF-1-regulated gene DEC1/Stra13: A mechanism for regulation of adipogenesis by hypoxia. *Developmental Cell* **2**, 331-341.

Zammit, P. S., Golding, J. P., Nagata, Y., Hudon, V., Partridge, T. A. and Beauchamp, J. R. (2004). Muscle satellite cells adopt divergent fates: a mechanism for self-renewal? *Journal of Cell Biology* **166**, 347-357.

Zammit, P. S., Relaix, F., Nagata, Y., Ruiz, A. P., Collins, C. A., Partridge, T. A. and Beauchamp, J. R. (2006). Pax7 and myogenic progression in skeletal muscle satellite cells. *Journal of Cell Science* **119**, 1824-1832.

Zhang, H. B., Ryu, D., Wu, Y. B., Gariani, K., Wang, X., Luan, P. L., D'Amico, D., Ropelle, E. R., Lutolf, M. P., Aebersold, R. et al. (2016). NAD(+) repletion improves mitochondrial and stem cell function and enhances life span in mice. *Science* **352**, 1436-1443.

Zhang, L., Zhou, F. F. and ten Dijke, P. (2013). Signaling interplay between transforming growth factor-beta receptor and PI3K/AKT pathways in cancer. *Trends in Biochemical Sciences* **38**, 612-620.

CHAPTER 2.   METHOD

2.1   Animals

All animal experiments are proved by Purdue University's Animal Care and Use

Committee. Mice were maintained in the Purdue Life Science Animal building. Mice

room temperature was set at 27°C and lighting was set on a 12 h light (6 a.m. to 6

p.m.)/dark (6 p.m. to 6 a.m.) cycle. Humidity and temperature were automatically

controlled. Mice were fed with autoclaved chow diet and clean water. Sick mice were

euthanized by CO_2 and death was confirmed by surgical spinal cord dislocation Purdue

Life Science Animal building. For experimental dissection, mice were sacrificed by

surgical spinal cord dislocation in Lab.

All mice used in this experiment were brought from Jackson Lab: Pax7$^{CreER/+}$

(#012476), Myod$^{Cre/+}$ (#014140), HIF1α$^{flox/flox}$ (#007561) and HIF2α$^{flox/flox}$ (#008407). All

mice were C57/B6 background. To obtain the MyodCre-HIFdKO and Pax7CreER-

HIFdKO mice, the following breeding strategy was used. MyodCre/+ mice were breeding

with HIF1af/f mice to get MyodCre/+ HIF1af/+ mice. MyodCre/+ HIF1af/+ mice were

breed with HIF2af/f mice to obtain MyodCre+/- HIF1af/+ HIF2af/+ and HIF1af/+

HIF2af/+mice. MyodCre+/- HIF1af/+ HIF2af/+ mice cross with HIF1af/+ HIF2af/+ mice

to obtain MyodCre-HIFdKO mice and control mice. Similar strategy was used to obtain

Pax7CreER-HIFdKO mice.

2.2   Genotyping

Genotypes were confirmed using PCR analysis according to instructions provided

by the Jackson Laboratory. The agents used for one 15μl PCR reaction system were:

13□l mix buffer (1.5µl 10X PCR buffer (Feldan), 0.3µl of 25mM dNTP (Feldan), 0.6µl

DMSO, 1.2µl primer mix (20pm each),0.075µl DNA polymerase (Bio Canada INC) and

9.5µl purified H$_2$O) mixed with 2µl DNA template. The primers used for genotyping

were listed in table below.

Table 1 Primers, anneal temperature and correspond size of band and genotype.

Target Gene	Primer Sequence	Anneal T	Size and Genotype
MyoDCre	CAC GAC TGC TTT CTT CAC CA	65	wild type 161 bp
	AAG TCT ATG TCC CGG AGT GG		mutant 320 bp
	CAA GGG ACA TCT TCC CAT TC		
Pax7CreER	GCT GCT GTT GAT TAC CTG GC	68	Mutant 235 bp
	CTG CAC TGA GAC AGG ACC G		Wild type 419 bp
	CAA AAG ACG GCA ATA TGG TG		
HIF1af/f	TGC ATG TGT ATG GGT GTT TTG	65	wild type 99 bp
	GAA AAC TGT CTG TAA CTT CAT TTC C		mutant 120 bp
HIF2af/f	GAG AGC AGC TTC TCC TGG AA	59	wild type 182 bp
	TGT AGG CAA GGA AAC CAA GG		mutant 220 bp

2.3 Conditional Knockout Induction

HIF1α and HIF2α deletion induction in animals:

The flox site HIF1$\alpha^{f/f}$HIF2$\alpha^{f/f}$ can be deleted after tamoxifen induction due to the Pax7CreER mice expressing tamoxifen inducible Cre-recombinase. Thus to induce HIF1α and 2α double knock-out in mice, we IP injected tamoxifen to mice every day for 5 days. For adult mice, 100μl tamoxifen/corn oil solution for each mouse is effective for inducing recombination. Tamoxifen solution was prepared as described below: Dissolve 500 mg of Tamoxifen in 5ml 100% ethanol in a 50 ml tube. After the tamoxifen was totally dissolved in ethanol, add 40 ml autoclaved sunflower oil. Protect the Falcon tube from light and spin and evaporate for 30 min. Separate the tamoxifen in 1.7 ml tubes, store at -20 C and protect from light.

Knockout induction in myoblasts:

HIF1/2a knocking out in myoblasts is induced by 4-OH-tamoxifen. Myoblasts were cultured in DMEM medium containing 4-OH-tamoxifen. After two days of induction, remove culture medium and wash cells with PBS. After cultured for two more days, check knocking out efficiency by mRNA level.

2.4 Muscle Injury and Regeneration

In general muscle injury is induced by cardiotoxin (CTX) injection. For tensor tibialis (TA) muscle, 50ul (10μM) CTX was injected into the middle of TA muscle. To ensure evenly injury, the needle was parallel injected into TA muscle. Slightly withdraw the needle as injection goes. For one mouse, left leg was injected with CTX to induce injury, another as control was injected with saline the same way. After 7 days of regeneration, muscle samples were collected and tested for the regeneration status.

2.5 C2C12 and OP9 Cell Line Culture

The classic myoblast cell line C2C12 were used for luciferase assay. OP9 cell line was used for induce continuously activation of Notch signaling. C2C12 and OP9 cells were cultured in DMEM with 10% FBS, 1% Penicillin / Streptomycin. Medium was changed every other day. Cells were passaged when reaching 80% confluent.

To continuously induce Notch signaling, primary myoblasts were co-cultured with 80% confluent OP9 cells in myoblast culture media (Gibco Ham's F-10 medium, 20% FBS, 1% penicillin/streptomycin P/S, 0.1% bFGF). After 2 days culture, analyze the cell state of primary myoblasts.

2.6 Primary Myoblast Isolation and Culture

Mouse hind limb muscles were collected, minced, and digested in 2.5ml of collagenase/dispase solution (Roche, 10ug/ml collagenase B, 2.4U/ml dispase in PBS) for 30 minutes. The digestion was stopped by myoblast culture media (Gibco Ham's F-10 medium, 20% FBS, 1% penicillin/streptomycin P/S, 0.1% bFGF). The digested medium was passed through a 100μm cell strainer and spun down at 450g for 5min. The pellets were suspended in culture medium and cultured at 37°C with 5% CO_2. Fresh medium was added daily during the first 3 days. Starting from day 3, medium was changed every other day until 70% confluence for 3 or 4 passages was achieved.

2.7 Single Fiber Isolation and Culture

Extensor digitorum longus (EDL) muscles from 10-week-old mice were isolated and digested in single fiber digestion solution (0.2% collagenase I in Dulbecco's Modified Eagle Medium or DMEM) for 30 minutes in 37°C water bath with gentle

shaking every 5 min. Single fibers were dissociated by gently pipetting with glass

pipettes and intact fibers were collected. To test satellite cell number, isolated single

fibers were transferred into fixative reagents (4% paraformaldehyde PFA solution) for

staining. For single fiber culture, isolated single fibers were transferred to horse serum

coated culture dish and cultured in culture media (Dulbecco's Modified Eagle's Medium,

20% FBS, 1% penicillin/streptomycin (P/S), 0.1% bFGF).

2.8 Hypoxia Treatment

To induce hypoxia, cells were cultured in a hypoxia chamber for 72 hours in 1% O_2

level. Gas was refilled every day. To mimic hypoxic conditions, cells were treated with

CoCl2 (final concentration 150 μM) for 6 hours.

2.9 Hematoxylin and Eosin (H&E) Staining

TA and Extensor digitorum longus (EDL) muscles were collected and submerged

in optimal cutting temperature compound (OCT) using dry ice-cold 2-methol-butane.

Cryosections (10 μm) were obtained and washed with PBS, emerged in Hematoxylin for

5min, rinsed with deionized water, and then incubated in eosin for 2 minutes. Slides were

then dried with sequential 75%, 95% and 100% alcohol (1min each), placed in Xylene for

5 min and mounted with mounting medium.

2.10 Immunohistochemistry

Muscle transversal cryosections, muscle fibers or cells obtained were fixed with

4% paraformaldehyde (PFA) solution for 10 minutes, incubated in 100mM glycine

solution and rinsed with PBS 3 times. The products were blocked in blocking buffer (5%

goat serum, 2% BSA, 0.2% triton X-100 and 0.1% sodium azide in PBS) for 1 hour at

room temperature, incubated in primary antibodies diluted with blocking buffer overnight

at 4°C and washed with PBS 3 times. PBS-diluted secondary antibodies conjugated with

4',6-diamidino-2-phenylindole (DAPI) were then applied for 1 hour at room temperature,

followed by 3 PBS rinses. Final products were mount with Dako fluorescent mounting

media (Glostrup, Denmark) and imaged with Coolsnap HQ CCD camera (photometrics,

USA) driven by IP Lab software (ScanalyticsInc, USA) using Leica DMI 6000B

fluorescent microscope. Primary antibodies: Pax7 1:20 (Developmental Studies

Hybridoma Bank), MyoD (Santa Cruz M-318, CA) 1:500. Secondary antibodies: 1:1000.

Quantification of myofiber size were conducted by ImageJ software.

2.11 Relative mRNA Level by qPCR

Quantitative realtime polymerase chain reaction (qPCR) analysis was performed

using a Roche Lightcycler 480 system. Total RNA was extracted by Trizol and 4 μg of

RNA was converted into cDNA using MMLV reverse transcriptase and random hexamer

primers. For each qPCR reaction, 5μl SYBR Green Mastermix (Roche), 0.2μl of each

primer (20μM) and 4.6μl cDNA (2ng/ul) were mixed. Relative fold changes were

calculated using 2-ΔΔCT method and 18s gene as a housekeeping control. All primers

used are listed below.

18S	AACGGTCTAGACAACAAGCTG AGTGGTCTTGGTGTGCTGAC
HIF1α	TCTCGGCGAAGCAAAGAGTC AGCCATCTAGGGCTTTCAGATAA
HIF2α	GTGCTCCCACGGCCTGTA TTGTCACACCTATGGCATATCACA
HES1	CCAGCCAGTGTCAACACGA AATGCCGGGAGCTATCTTTCT
HES2	CTGAAGGGTCTCGTATTGCCG CGCAGGTGCTCTAGTAGGC
HES3	GCACGCATCAACGTGTCAC TGAGTTCTGGAGGCTTCTCAT
HEY2	AAGCGCCCTTGTGAGGAAAC GGTAGTTGTCGGTGAATTGGAC
HEYL	CAGCCCTTCGCAGATGCAA CCAATCGTCGCAATTCAGAAAG
HES6	ACCACCTGCTAGAATCCATGC GCACCCGGTTTAGTTCAGC

2.12 Western Blot

Total protein was isolated from cells using RIPA buffer (pH8.0 50mM Tris-HCL, 150mMNaCl, 1%NP-40, 0.5% sodium deoxycholate and 0.1% SDS). Concentrations were determined by BCA Protein Assay Reagent (Pierce Biotechnology, Rockford, IL). Proteins adjusted to the same concentrations and volumes were separated by sodium dodecyl sulfate PAGE (SDS-PAGE), transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore Corporation, Billerica, MA), blocked with 4% milk, and incubated with the diluted primary antibodies at 4°C overnight. HIF1α primary antibodies (NB100-

105, Novus Biologicals, IL) were used with 1:500 dilutions, NICD primary antibodies (ab8925, Abcam) were used as 1:2000, α-tubuline primary antibodies (B-7, Santa Cruz, CA) were used as 1:1000 dilution. Band detection was performed using enhanced chemiluminescence (ECL) western blotting substrate (Pierce Biotechnology, Rockford, IL) and detected with a FluorChem R protein imaging system (Protein Simple, CA, USA).

2.13 Image Analysis

Protein intensity was captured from western blot by NIH imageJ software. For each protein, the intensity was measured from each band using gel analysis method. Relative protein content was calculated as each protein intensity divided by α-tubulin intensity. Statistical analysis was used for relative protein content comparison.

2.14 Statistics Analysis

Data are presented as mean ± standard error mean (SEM). Numbers represent biological replicates unless indicated. P-values were calculated using two-tailed student t-test (* $P<0.05$, ** $P<0.01$, ***$p<0.005$).

CHAPTER 3.　　RESULTS

3.1　Myod^{cre} Mediated Double Knockout of HIF1α and HIF2α does Not Affect Muscle Development

Previous studies have shown that HIF1α are indispensable for embryonic

development, and global loss of HIF1α leads to lethality (Forristal et al., 2010; Iyer et

aliningn., 1998; Simon and Keith, 2008). HIF2α deficient mice develop severe vascular

defects and show developmental arrest between E9.5 and E12.5 with variability

depending on the genetic background (Compernolle et al., 2002; Forristal et al., 2010).

Hence, the tissue–specific function of HIFs in muscle development remains unclear. As

Pax3^{Cre}-mediated specific deletion of HIF1α in embryonic satellite cells results in

apparently normal skeletal muscles (Majmundar et al., 2015), we sought to examine

whether HIF1α and HIF2α play redundant role in muscle development. To achieve this,

we developed the HIF1α and HIF2α double knockout mouse model using the muscle-

specific Myod^{Cre} as a driver (Myod-HIF^{dKO}) (Figure 1). Since Myod is specifically and

ubiquitously activated in proliferating myoblasts, this model should result in deletion of

HIF1α and HIF2α in muscle progenitors and mature myofibers (Collins et al., 2005;

Zammit et al., 2004). We designed primers to confirm specific and efficient knock out

(Figure 1). Indeed, analysis of the amplified mRNA size and relative mRNA levels

confirmed the specific and efficient knockout of HIFs in the skeletal muscles (Figure 2,

3).

　　　Surprisingly, the Myod-HIF^{dKO} mice were born at normal Mendelian ratio and did

not exhibit any morphological abnormality. Specifically, the initial body weight and

postnatal growth of Myod-HIF^{dKO} mice were completely normal (Figure 5). Furthermore,

the weight and size of various muscles were identical between control and Myod-HIF$^{\text{dKO}}$ mice (Figure 6). Muscle morphology was also indistinguishable between the two groups (Figure 7). Finally, we measured myofiber number and size of EDL muscles and did not find any differences between control and Myod-HIF$^{\text{dKO}}$ mice (Figure 8). These results suggest that HIF1α and HIF2α are dispensable for skeletal muscle development and postnatal growth under normal oxygen conditions.

3.2 HIF1α and HIF2α Deficiency Impedes Muscle Regeneration

The normal development and growth of skeletal muscles in the MyoD-HIF$^{\text{dKO}}$ mice suggest that coordinated angiogenesis and myogenesis may have ensured adequate oxygen supply and rendered HIF1α and HIF2α dispensable for embryonic myogenesis. By contrast, ischemic low oxygen levels (hypoxia) typically occur following muscle injury and during muscle regeneration (Merrick, 2002). Indeed, examination of HIF1α and HIF2α expression indicates that HIF1α protein and mRNA levels rise following cardiotoxin (CTX)-induced muscle injury, peaking at 2–3 days post injury (DPI) when active myoblast proliferation occurs (Figure 9, 10). However, HIF2α levels decrease dramatically upon injury-induced myofiber degeneration and gradually return to the normal level at the completion of muscle regeneration (Figure 11). The in vivo dynamics of HIF1α and HIF2α during muscle regeneration suggest that HIF1α is mainly expressed in myoblasts but HIF2□ is mainly expressed in myofibers. Consistent with this notion, HIF1α expression is much higher than that of HIF2α in proliferating myoblasts, but HIF2α expression is much higher than that of HIF1α in post-differentiation myotubes (Figure 12). These results suggest that regenerative muscles are challenged by hypoxia,

and HIF1α and HIF2α may play stage-specific functions in myoblasts and myofibers, respectively.

To identify the role of HIF1α and HIF2α in satellite cell-mediated muscle regeneration, we used satellite cell specific Pax7[CreER] to drive double knockout of HIF1α and HIF2α (Pax7[CreER]-HIF[dKO]) (Halevy et al., 2004; Olguin and Olwin, 2004; Zammit et al., 2004) (Figure 13). In this model, HIF1α and HIF2α should be specifically knocked out in satellite cells after tamoxifen induction (IP injection). The control group includes HIF1α[f/f]HIF2α[f/f] mice similarly injected with tamoxifen. To confirm the efficiency of tamoxifen induced knockout, we measured the HIF1α and HIF2α mRNA level in satellite cells isolated from tamoxifen induced mice. This analysis showed that HIF1α and HIF2α were efficiently knocked out in myoblasts (Figure 14).

Following tamoxifen-induced deletion of HIF1α and HIF2α in satellite cells, CTX was injected into TA muscle to induce muscle regeneration in both control and Pax7[CreER]-HIF[dKO] mice. Samples were collected 7 DPI (Figure 15). Compared with those of control mice, the CTX-injected TA muscles of Pax7[CreER]-HIF[dKO] mice exhibited a decreased size and a significantly lower muscle weight (Figure 15, 18). Compared to the control muscles, TA muscles of Pax7[CreER]-HIF[dKO] mice were poorly regenerated, manifested by fewer newly regenerated, central nucleated myofibers but increased infiltration of non-muscle cells (Figure 16, 17). We also measured the regenerated and non-regenerated areas from cross sections of control mice and Pax7[CreER]-HIF[dKO] mice. Pax7[CreER]-HIF[dKO] mice exhibited a lower regenerative area as well as a higher non-regenerative region (Figure 19). Thus, we conclude that HIF1α and HIF2α deficiency impairs muscle regeneration.

3.3 HIF1α and HIF2α Deficiency Decrease Satellite Cell Number during Muscle Regeneration

As satellite cells are the main driving force of muscle regeneration (Kuang and Rudnicki, 2008), we next investigated if defective muscle regeneration is associated with reduced satellite cell number or function. To assess the satellite cell numbers during regeneration, we used Pax7 as a satellite cell marker (Seale et al., 2000). Pax7 positive cells were measured both in normal muscle and regenerative muscle from Pax7^{CreER}-HIF^{dKO} and control mice. Judging from TA cross-sections (Figure 20), Pax7^{CreER}-HIF^{dKO} mice showed a significantly decreased satellite cell numbers compared with control mice at 7 DPI (Figure 22), while the satellite cell numbers in normal muscle are identical (Figure 22). Therefore, we hypothesized that the impaired muscle regeneration may be a consequence of the reduced satellite cell number during regeneration. To confirm our hypothesis, we also counted satellite cells in fresh–isolated regenerated myofibers (Figure 21). Consistent with the cross-section results, a significantly decreased satellite cell number was observed on the myofibers (Figure 23). We conclude that HIF1α and HIF2α deficiency impairs muscle regeneration by decreasing satellite cell number.

3.4 HIF1α and HIF2α Deficiency Inhibits Self-Renewal But Promotes Differentiation of Satellite Cells

Satellite cells undergo self-renewal to maintain the stem cell pool (Relaix and Zammit, 2012; Wang et al., 2014b). We hypothesized that the decreased satellite cell population in Pax7^{CreER}-HIF^{dKO} mice may be due to insufficient self-renewal. To test this possibility, we assessed the self-renewal and differentiation ratio in HIF1α/2α dKO and control myoblast under hypoxia. To induce the knockout of HIF1α and HIF2α in cultured myoblasts, we isolated myoblasts from Pax7^{CreER}-HIF1α$^{f/f}$HIF2α$^{f/f}$ and

HIF1$\alpha^{f/f}$HIF2$\alpha^{f/f}$ mice, and then treated the primary myoblasts with 4-hydroxyl tamoxifen (4-OH-TMX) for 2 days. After 2 days removal of 4-OH-TMX, we confirmed an efficient knockout as described before.

At 72 h after hypoxia (1% O2) treatment, we used a well-documented method to classify cell status as self-renewed (Pax7$^+$/MyoD$^-$), proliferating (Pax7$^+$/MyoD$^+$) and differentiating (Pax7$^-$/MyoD$^+$) (Figure 24) (Halevy et al., 2004; Olguin and Olwin, 2004; Zammit et al., 2004). Under normoxia condition (21% O$_2$), no difference in cell status distribution was observed between the two groups (Figure 25). However, under the hypoxic state, HIF1α/2α dKO myoblast showed a significantly reduced self-renewal ratio and a relatively higher level of differentiation (Figure 26). Consistent with our previous results (Liu et al., 2012b), increased self-renewal was found in the hypoxia group compared with the normoxia group in control myoblasts. However, the hypoxia-enhanced self-renewal was largely diminished by HIF1α and HIF2α dKO, indicating that hypoxia promotes self-renewal through HIF1α and HIF2α.

Muscle satellite cells natively reside underneath the basal lamina, surrounded by the membrane of myofiber and basal lamina, forming a microenvironment (Katz and Miledi, 1961). To mimic the impact of hypoxia on satellite cell self-renewal in a physiological microenvironment, we isolated single myofibers from Pax7^{CreER}-HIF^{dKO} and HIF1$\alpha^{f/f}$HIF2$\alpha^{f/f}$ mice. After culturing under either hypoxia or normoxia condition for 72 hours, cultured single fibers were fixed for cell cycle state assay (Figure 27). Under normoxia state, the proliferating satellite cells number showed no significant difference between control and HIFdKO myofibers (Figure 28). In addition, the self-renewal and differentiating ratio is similar between control and HIFdKO myofibers

(Figure 29). However, control myofiber exhibited more proliferated myoblast and more myoblast cluster under hypoxia condition (Figure 30). Also, consistent with our primary myoblast culture results, under the hypoxic state, HIFdKO single fibers showed an increased ratio of differentiating and lower ratio of self-renewal compared with control myofibers (Figure 31).

It has been wildly accepted that hypoxia can inhibit myoblast differentiation. However whether HIF1/2α participates in inhibition of myoblast differentiation is still unclear. Thus, we induced control and Pax7Cre-HIFdKO myoblast differentiation under hypoxia to investigate the function of HIF1/2α in hypoxia induced differentiation inhibition. After 1.5 days induction culture in hypoxia, MF20 staining were used for demonstrating new formed myotube (Figure 32). To test the myoblast differentiation rate, we quantified myoblast fusion index. We found a significantly promoted myoblast differentiation and myotube formation, which indicates that HIF1α and HIF2α deficiency promotes satellite cell differentiation in hypoxia (Figure 33).

Thus we conclude that HIF1α and HIF2α deficiency inhibits satellite cell self-renewal and promotes their differentiation under the hypoxic state.

3.5 HIF1α and HIF2α Promote Satellite Cells Self-Renewal through Notch Signaling

Previous studies have shown that hypoxia activates Notch signaling in myoblasts, and subsequently promotes self-renewal and inhibits differentiation (Gustafsson et al., 2005a; Liu et al., 2012b). We hence hypothesized that HIF1α and HIF2α may promote satellite cell self-renewal through enhancing Notch signaling. To determine how HIF1α and HIF2α affect Notch signaling, we induced deletion of HIF1α and HIF2α by

adenovirus–Cre/GFP transduction into HIF1$\alpha^{f/f}$HIF2$\alpha^{f/f}$ myoblasts. Adenovirus–GFP was used in parallel as a control. After 24 hours of adenovirus infection, Successful HIF1/2α knockout was evident in the significantly reduced expression of HIF1α and HIF2α mRNA levels (Figure 34). Also, we observed a dramatically decreased HIF1α protein level under hypoxia, indicating a successfully induced knockout (Figure 37).

We next evaluated Notch activation based on the level of Notch1 intercellular domain (NICD), which is proteolytically cleaved following Notch activation (Conboy and Rando, 2002b; Kopan et al., 1994). Under normoxia conditions, NICD levels were similar between HIF^{dKO} and control groups (Figure 35, 36), suggesting that HIF1α and HIF2α do not affect Notch signaling under normoxia. However, after 6 hours $CoCl_2$ treatment (to mimic hypoxia), NICD level in HIF1α/2α dKO group was lower level than that in the HIF1$\alpha^{f/f}$ HIF2$\alpha^{f/f}$ group (Figure 37, 39). These results suggest that deletion of HIFs blunts hypoxia-induced activation of Notch signaling in myoblasts. To further confirm this notion, we examined the expression of Notch target genes. In the control myoblasts, Hes2 and Hey2 were significantly increased, whereas Hes6 was dramatically decreased by $CoCl_2$ treatment, indicating that hypoxia activates Notch signaling (Figure 40). But in the HIF1α/2α dKO group, $CoCl_2$ treatment had no effects on the expression of Notch target genes (Figure 41). Furthermore, whereas $CoCl_2$ treatment significantly increased the level of Pax7 in control myoblasts, it had no effect on Pax7 expression in HIF1α and HIF2α deficient myoblasts (Figure 42). These results demonstrate that HIF1α and HIF2α are required for hypoxia-induced activation of Notch signaling and upregulation of Pax7, the key transcriptional determinant of muscle stem cell fate.

3.6 Constitutive Activation of Notch Signaling Rescues HIFdKO Induced Inhibition of Satellite Cells Self-Renewal

To further confirm our hypothesis that hypoxia induced HIF1/2α promote satellite cells self-renewal through Notch signaling, we tested whether constitutive activation of Notch signaling can rescue HIFdKO induced inhibition of satellite cells self-renewal.

To constitutive activate Notch signaling in myoblast, we co-cultured HIFdKO and control myoblast with OP9 cells (Lehar et al., 2005). It has been demonstrated that OP9 cell lines highly express Dll1 (Notch ligands). Notch signaling is activated when Notch receptors binding with Notch ligands. Cells co-cultured with OP9 cells would be Notch signaling constitutive activated. After 72 hours hypoxia co-culture, we tested myoblast cell status as described before (Figure 43). Consistent with our hypothesis, HIFdKO myoblast exhibited similar self-renewal and differentiating ratio with control myoblast (Figure 44). Which means the HIFdKO induced inhibition of self-renewal is polished by activation of Notch signaling. Thus, we can conclude that HIF1/2α can promote satellite cells through Notch.

3.7 References

Collins, C. A., Olsen, I., Zammit, P. S., Heslop, L., Petrie, A., Partridge, T. A., and Morgan, J. E. (2005) Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. *Cell* **122**, 289-301

Compernolle, V., Brusselmans, K., Acker, T., Hoet, P., Tjwa, M., Beck, H., Plaisance, S., Dor, Y., Keshet, E., Lupu, F., Nemery, B., Dewerchin, M., Van Veldhoven, P., Plate, K., Moons, L., Collen, D., and Carmeliet, P. (2002) Loss of HIF-2alpha and inhibition of VEGF impair fetal lung maturation, whereas treatment with VEGF prevents fatal respiratory distress in premature mice. *Nat Med* **8**, 702-710

Conboy, I. M., and Rando, T. A. (2002) The regulation of notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. *Developmental Cell* **3**, 397-409

Forristal, C. E., Wright, K. L., Hanley, N. A., Oreffo, R. O. C., and Houghton, F. D. (2010) Hypoxia inducible factors regulate pluripotency and proliferation in human embryonic stem cells cultured at reduced oxygen tensions. *Reproduction* **139**, 85-97

Gustafsson, M. V., Zheng, X., Pereira, T., Gradin, K., Jin, S., Lundkvist, J., Ruas, J. L., Poellinger, L., Lendahl, U., and Bondesson, M. (2005) Hypoxia requires notch signaling to maintain the undifferentiated cell state. *Dev Cell* **9**, 617-628

Halevy, O., Piestun, Y., Allouh, M. Z., Rosser, B. W., Rinkevich, Y., Reshef, R., Rozenboim, I., Wleklinski-Lee, M., and Yablonka-Reuveni, Z. (2004) Pattern of Pax7 expression during myogenesis in the posthatch chicken establishes a model for satellite cell differentiation and renewal. *Dev Dyn* **231**, 489-502

Iyer, N. V., Kotch, L. E., Agani, F., Leung, S. W., Laughner, E., Wenger, R. H., Gassmann, M., Gearhart, J. D., Lawler, A. M., Yu, A. Y., and Semenza, G. L. (1998) Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. *Genes Dev* **12**, 149-162

Katz, B., and Miledi, R. (1961) The localized action of end-plate drugs' in the twitch fibres of the frog. *J Physiol* **155**, 399-415

Kopan, R., Nye, J. S., and Weintraub, H. (1994) The Intracellular Domain of Mouse Notch - a Constitutively Activated Repressor of Myogenesis Directed at the Basic Helix-Loop-Helix Region of Myod. *Development* **120**, 2385-2396

Kuang, S., and Rudnicki, M. A. (2008) The emerging biology of satellite cells and their therapeutic potential. *Trends Mol Med* **14**, 82-91

Lehar, S. M., Dooley, J., Farr, A. G., and Bevan, M. J. (2005) Notch ligands delta1 and jagged1 transmit distinct signals to T-cell precursors. *Blood* **105**, 1440-1447

Liu, W. Y., Wen, Y. F., Bi, P. P., Lai, X. S., Liu, X. S., Liu, X. Q., and Kuang, S. H. (2012) Hypoxia promotes satellite cell self-renewal and enhances the efficiency of myoblast transplantation. *Development* **139**, 2857-2865

Majmundar, A. J., Lee, D. S. M., Skuli, N., Mesquita, R. C., Kim, M. N., Yodh, A. G., Nguyen-McCarty, M., Li, B., and Simon, M. C. (2015) HIF modulation of Wnt signaling regulates skeletal myogenesis in vivo. *Development* **142**, 2405-+

Merrick, M. A. (2002) Secondary injury after musculoskeletal trauma: a review and update. *J Athl Train* **37**, 209-217

Olguin, H. C., and Olwin, B. B. (2004) Pax-7 up-regulation inhibits myogenesis and cell cycle progression in satellite cells: a potential mechanism for self-renewal. *Developmental Biology* **275**, 375-388

Relaix, F., and Zammit, P. S. (2012) Satellite cells are essential for skeletal muscle regeneration: the cell on the edge returns centre stage. *Development* **139**, 2845-2856

Seale, P., Sabourin, L. A., Girgis-Gabardo, A., Mansouri, A., Gruss, P., and Rudnicki, M. A. (2000) Pax7 is required for the specification of myogenic satellite cells. *Cell* **102**, 777-786

Simon, M. C., and Keith, B. (2008) The role of oxygen availability in embryonic development and stem cell function. *Nat Rev Mol Cell Bio* **9**, 285-296

Wang, Y. X., Dumont, N. A., and Rudnicki, M. A. (2014) Muscle stem cells at a glance. *J Cell Sci* **127**, 4543-4548

Zammit, P. S., Golding, J. P., Nagata, Y., Hudon, V., Partridge, T. A., and Beauchamp, J. R. (2004) Muscle satellite cells adopt divergent fates: a mechanism for self-renewal? *J Cell Biol* **166**, 347-357

CHAPTER 4.   SUMMARY AND DISCUSSION

4.1   Discussion

Given the important role that HIF1α plays in embryonic development, we expected to identify their specific functions in muscle satellite cells. However, previous studies have shown that Pax3-triggered satellite cell specific HIF1α knockout not alter embryonic muscle development (Majundar et al., 2015). The inconsistency between expectations and observations made us hypothesize that the complementary function of HIF2α may rescue HIF1α deficiency. However, our results demonstrated that Myod-HIF^{dKO} mice exhibited normal myofiber size and number in adults, suggesting that HIF1α and HIF2α are dispensable for normal muscle development. However, whether HIF3α plays a compensatory role in the absence of HIF1α and HIF2α has yet to be determined.

Following muscle injury, skeletal muscle progenitor cells are challenged by hypoxia due to combined reduction of oxygen supply (caused by degeneration of blood vessels) and higher oxygen (or energy) demand during regeneration (Merrick, 2002). Our data, consistent with previous studies, shows that HIF1α expression is elevated during muscle regeneration, while HIF2α is initially decreased but slowly increases during regeneration. These results indicate that HIFs may be necessary for muscle regeneration. As expected, in the CTX-induced muscle regeneration model, HIF1α and HIF2α deficient mice exhibited impaired muscle regeneration and reduced satellite cell number. Further, our *in vitro* study demonstrates that hypoxia promote satellite cell self-renewal in wildtype myoblasts. While HIF1α and HIF2α deficiency diminishes the effect of hypoxia on self-renewal, indicating that hypoxia-enhanced self-renewal is largely dependent on HIF1α and HIF2α.

Our previous research has demonstrated that hypoxia promotes satellite cell self-renewal and enhances the efficiency of myoblast transplantation (Liu et al., 2012b). Additionally, the hypoxia-induced enhancement of self-renewal was highly dependent on Notch signaling activation. Hypoxia also activates Notch signaling in other tissues (Clarke and van der Kooy, 2009; Eliasson and Jonsson, 2010). In the present study, we confirmed our previous results, and furthermore, we found that hypoxia-induced activation of Notch is dependent on HIF1α and HIF2α, which are stabilized by hypoxia. Upon the double knockout of HIF1α and HIF2α, hypoxia fail to upregulate NICD, pointing to the critical function of HIF1α and HIF2α in mediating the effect of hypoxia on Notch signaling. In this regard, the diminished promotion of Notch signaling abolishes the hypoxia-induced upregulation of Pax7 and further decreased satellite cell self-renewal in the HIF1α2α dKO myoblasts.

In the present study, we demonstrate that the HIF-Notch axis controls satellite cell self-renewal in adult muscle regeneration. For the first time, we show that injury-induced increase of HIF1α and HIF2α contributes to the maintenance and long-term homeostasis of satellite cells by promoting their self-renewal. Interestingly, HIF1α and HIF2α are both dispensable for embryonic myogenesis. There are two potential explanations for this. First, even though developing embryos are exposed to a hypoxic environment, all cells in the embryo are not under the same hypoxic condition. It has been reported that cells in various regions of the embryo are challenged by different levels of hypoxia (Ream et al., 2008). Thus, embryonic muscle progenitors may be sufficiently oxygenated to render HIFs dispensable. As HIFs mediate hypoxia-induced activation of Notch signaling, an alternative possibility is that embryonic myoblasts exhibit sufficiently high level of Notch

signaling even in the absence of HIF1α and HIF2α. Indeed, it has been shown that muscle progenitors in embryonic stage intrinsically express high level of Notch (Vasyutina et al., 2007). During injury, however, a temporal switch from Notch to Wnt signaling is required for normal adult myogenesis (Brack et al., 2008). Hence, hypoxia-induced activation of Notch may switch a subpopulation of activated satellite cells to self-renewal, preventing excessive myogenic differentiation and maintaining a sustainable pool of satellite cells. Our finding broadens the understanding of the mechanisms underlying the microenvironment and oxygen level involved in the muscle regeneration process and provides novel insights into therapies for muscle diseases and improving muscle function.

4.2　Future Research

In the present study, we clearly demonstrated that HIF1α and HIF2α double knockout impedes adult muscle regeneration through inhibition of Notch signaling. However, there are two important but still unclear questions yet to be answered.

The first question is: Given the critical roles of HIFs in embryonic development, why double knockout of HIF1α and HIF2α does not impact muscle development? One possibility is that HIF1α and HIF2α may impact embryonic muscle development only when the oxygen is under certain level. As described before, the significant impact of hypoxia on myoblast was observed only when oxygen level is less than 2%. The fact is that although the oxygen level in embryo is lower than that in postnatal individuals, it is still high enough which may not be able to trigger the hypoxia reactions in muscle. To investigate the role of HIF1α and HIF2α in embryonic muscle development, what could be done is to challenge pregnant mice with hypoxia to induce even lower oxygen level in

embryo. By comparing the development of normal and HIF1α/HIF2α deficient muscle under severe hypoxia state, the function of HIF1α and HIF2α in embryonic muscle development could be further determined.

The second question is: What is the function of HIF3α in satellite cells? Because of lack the mice model, we were unable to test whether HIF3α deficiency affects muscle and satellite cell function. HIF3α is different from HIF1α and HIF2α. As far as concerned, HIF1α and HIF2α function similarly but not identically. However, HIF3α acts as an inhibitor of HIF1α and HIF2α through competing with HIF1α and HIF2α for HIF1β binding, but drives HIF1β to different promoter regions. Thus, high expression of HIF3α can somehow blunt the function of HIF1α and HIF2α. To test the function of HIF3α in satellite cell, we could first investigate the impact of HIF3α deficiency on cultured myoblast. Further we could introduce HIF3α knockout mice model to test its function *in vivo*. With this regard, still lots of myth need to be classified in terms of the function of HIFs in muscle and satellite cell function.

4.3   Reference

Brack, A. S., Conboy, I. M., Conboy, M. J., Shen, J. and Rando, T. A. (2008). A temporal switch from Notch to Wnt signaling in muscle stem cells is necessary for normal adult myogenesis. *Cell Stem Cell* **2**, 50-59.

Clarke, L. and van der Kooy, D. (2009). Low Oxygen Enhances Primitive and Definitive Neural Stem Cell Colony Formation by Inhibiting Distinct Cell Death Pathways. *Stem Cells* **27**, 1879-1886.

Eliasson, P. and Jonsson, J. I. (2010). The Hematopoietic Stem Cell Niche: Low in Oxygen but a Nice Place to be. *Journal of Cellular Physiology* **222**, 17-22.

Liu, W. Y., Wen, Y. F., Bi, P. P., Lai, X. S., Liu, X. S., Liu, X. Q. and Kuang, S. H. (2012). Hypoxia promotes satellite cell self-renewal and enhances the efficiency of myoblast transplantation. *Development* **139**, 2857-2865.

Majmundar, A. J., Lee, D. S. M., Skuli, N., Mesquita, R. C., Kim, M. N., Yodh, A. G., Nguyen-McCarty, M., Li, B. and Simon, M. C. (2015). HIF modulation of Wnt signaling regulates skeletal myogenesis in vivo. *Development* **142**, 2405-+.

Merrick, M. A. (2002). Secondary injury after musculoskeletal trauma: a review and update. *J Athl Train* **37**, 209-17.

Ream, M., Ray, A. M., Chandra, R. and Chikaraishi, D. M. (2008). Early fetal hypoxia leads to growth restriction and myocardial thinning. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology* **295**, R583-R595.

Vasyutina, E., Lenhard, D. C., Wende, H., Erdmann, B., Epstein, J. A. and Birchmeier, C. (2007). RBP-J (Rbpsuh) is essential to maintain muscle progenitor cells and to generate satellite cells. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 4443-4448.

4.4 Figures
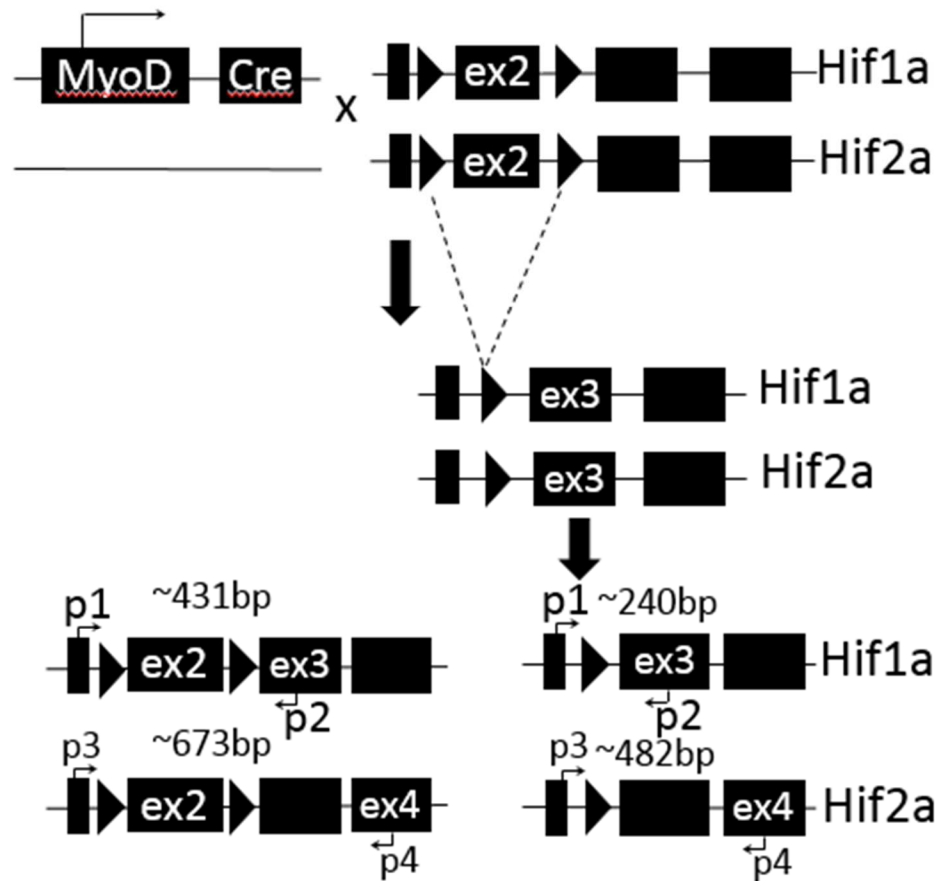
Figure 1. Breeding and validation strategy of MyodCre-HIFdKO mice.
HIF1/2α $^{\text{flox/flox}}$ mice cross with MyoDCre mice to get MyoDCre-HIF$^{\text{dKO}}$ mice. Primers were designed in the first exon and the third exon to validate knock out efficiency.
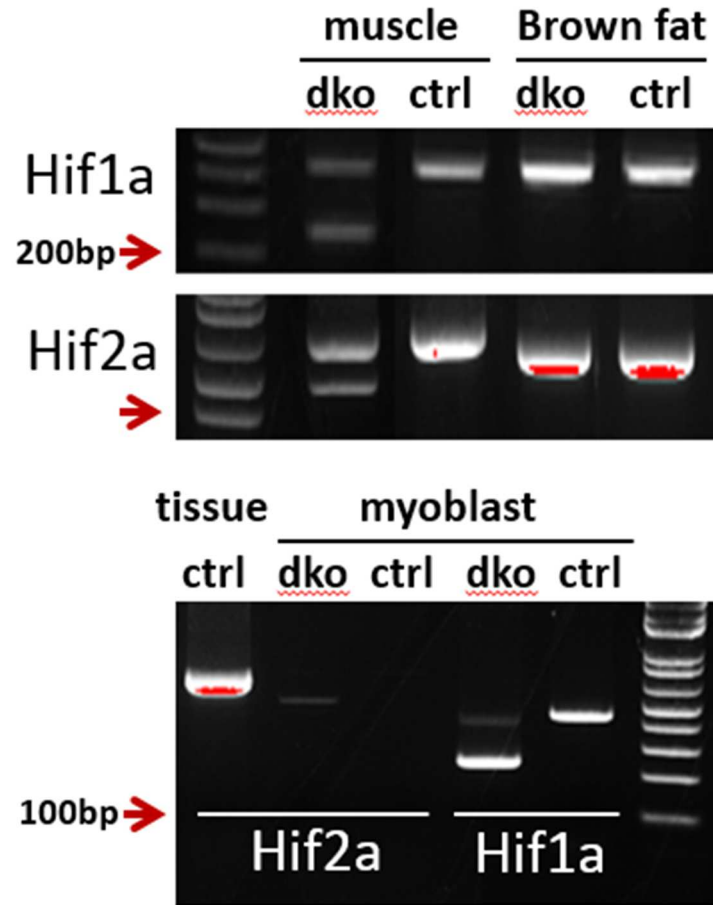
Figure 2. Validation of HIF1/2α double knockout.

MyoDCre negative HIF1/2α $^{flox/flox}$ mice were used as control as well as brown fat. RNA was extract from tissue and myoblast to generate cDNA. Target piece of cDNA were amplified by PCR. MyoD-HIFdKO mice showed a lower knocking out band. (Figures provided by Xin Yang.)
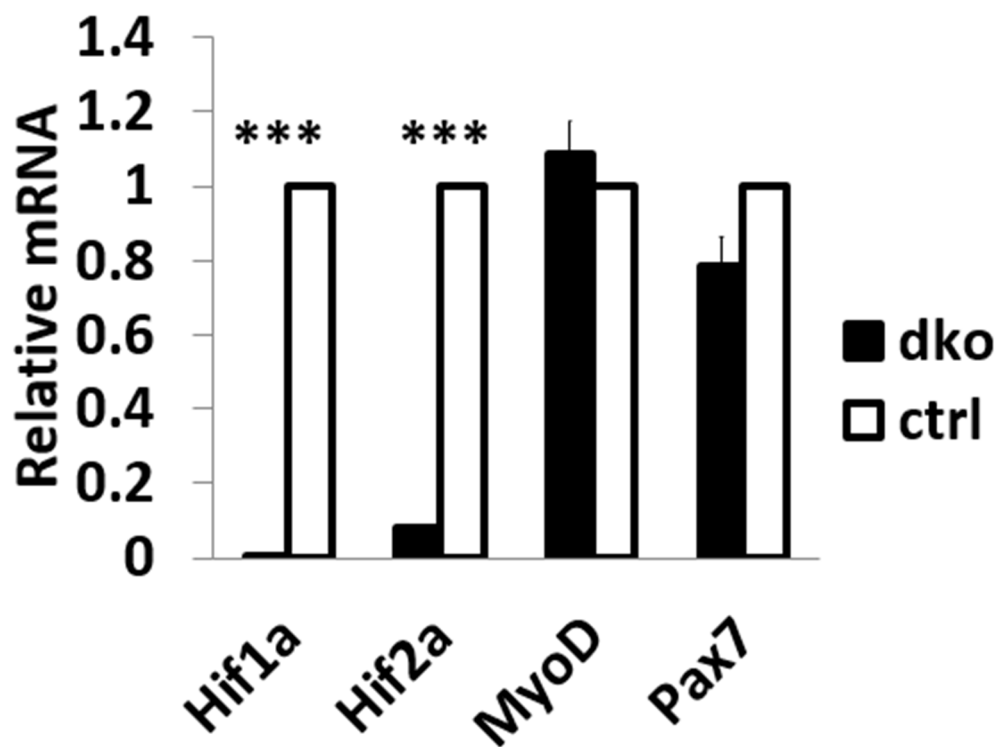
Figure 3. Validation of knocking out Hif1α and Hif2α by mRNA content.

Total RNA isolated from myoblasts was used for validating HIF1/2 α knocking out.

HIF1α and HIF2α mRNA showed a significant decrease in knock out group compared to

control. (3 pair of Control and HIFdKO myoblast were used for student t test analysis,

***p<0.005)
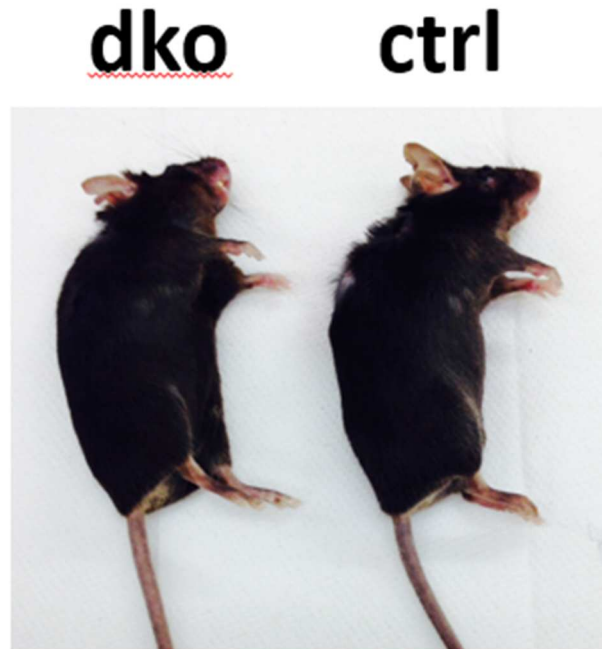
Figure 4. Representative picture of MyodCre HIF^{dKO} mice and control mice.
In terms of size, Myod-HIF^{dKO} mice showed no significant difference with control mice.
(Figures are provided by Xin Yang)
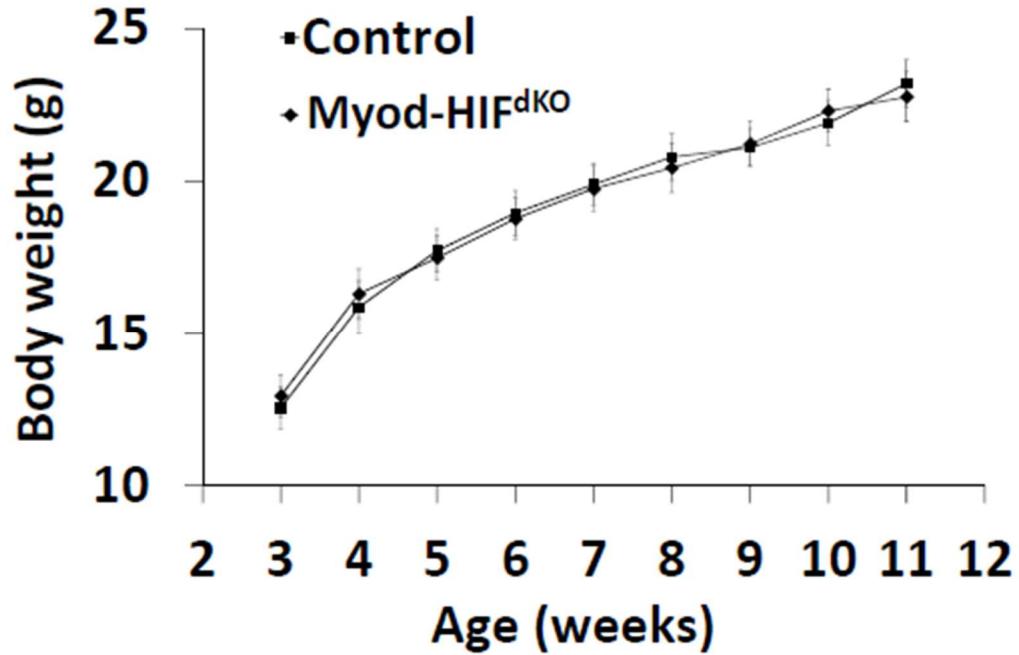
Figure 5. Post-natal growth curve of Myod-HIFdKO and control mice.
Myod-HIF^{dKO} (n=8) mice and Control (n=10) mice were weighed weekly beginning at weaning, and showed no difference in body weight across the 12 weeks of analysis. (Figures are provided by Xin Yang)
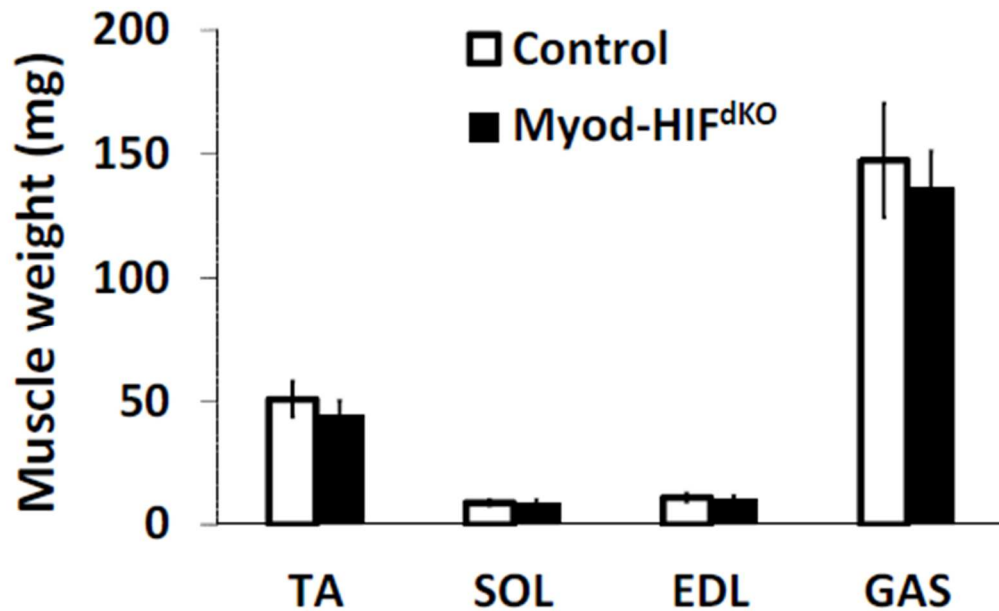
Figure 6 Muscle weight of Myod-HIFdKO mice and Control mice.

At 12 weeks of age tendon tibialis (TA), soleus (SOL), extensor digitorum longus (EDL), and gastrocnemius muscle (GAS) were dissected Myod-HIF^{dKO} mice (n=5) and Control (n=5) mice and weighed. There was no significant difference in muscle weight between the treatments. (Figures are provided by Xin Yang)
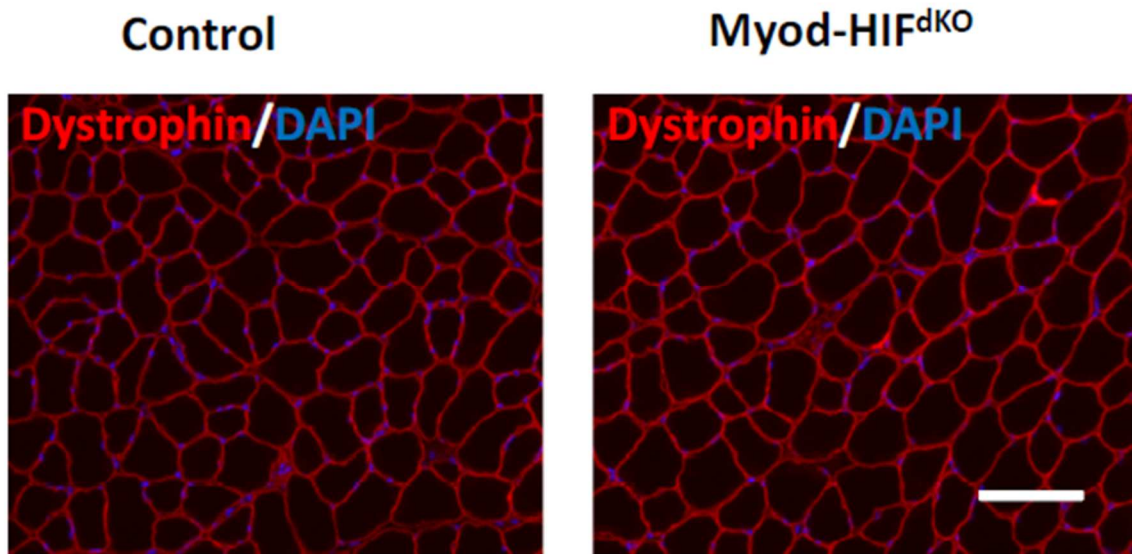
Figure 7. Representative picture of dystrophin staining of TA muscle from control and Myod-HIFdKO mice cross-section.

TA muscle was dissected from 10 week-old mice, submerged in OCT, plunged in liquid nitrogen and cryosectioned. Sections from A) control and B) Myod-HIF muscle tissue were stained with distrophin (red) issue DAPI (blue) to visualize muscle fibers. Images were used to measure muscle fiber size and number. Dystrophin staining was used to quantify muscle fiber size and number.
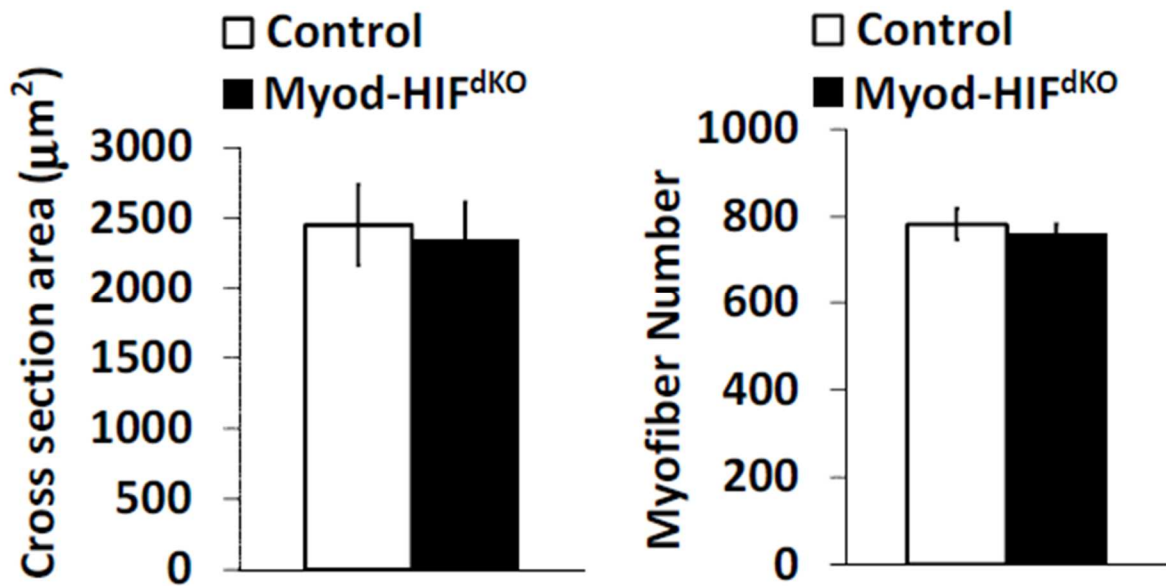
Figure 8. Quantification of myofiber size and number.

Myofiber size was quantified in cross-sections of TA muscles (Fig. 7) using imageJ software. Myofiber number was quantified by total number in each cross-section. Myod-HIF^{dKO} mice did not show any significant difference in terms of myofiber size and number. (Samples taken from 5 Myod-HIF^{dKO} and 5 control mice were analyzed for statistics.)
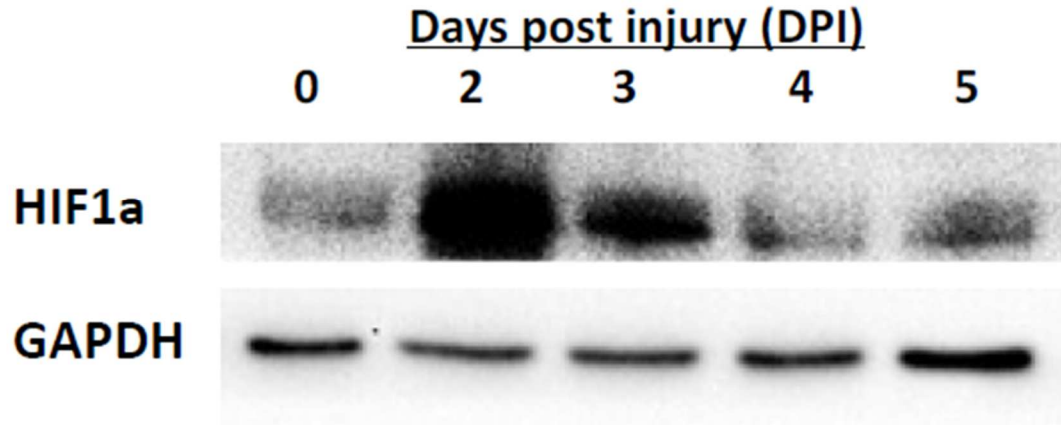
Figure 9. Western blot of HIF1α level during regeneration of TA muscle. Injury TA muscle was dissected from mice (n=5). HIF1α content was significantly increased after injury and reached peak 2 days post injury. As regeneration goes on, HIF1α protein level returns to normal.
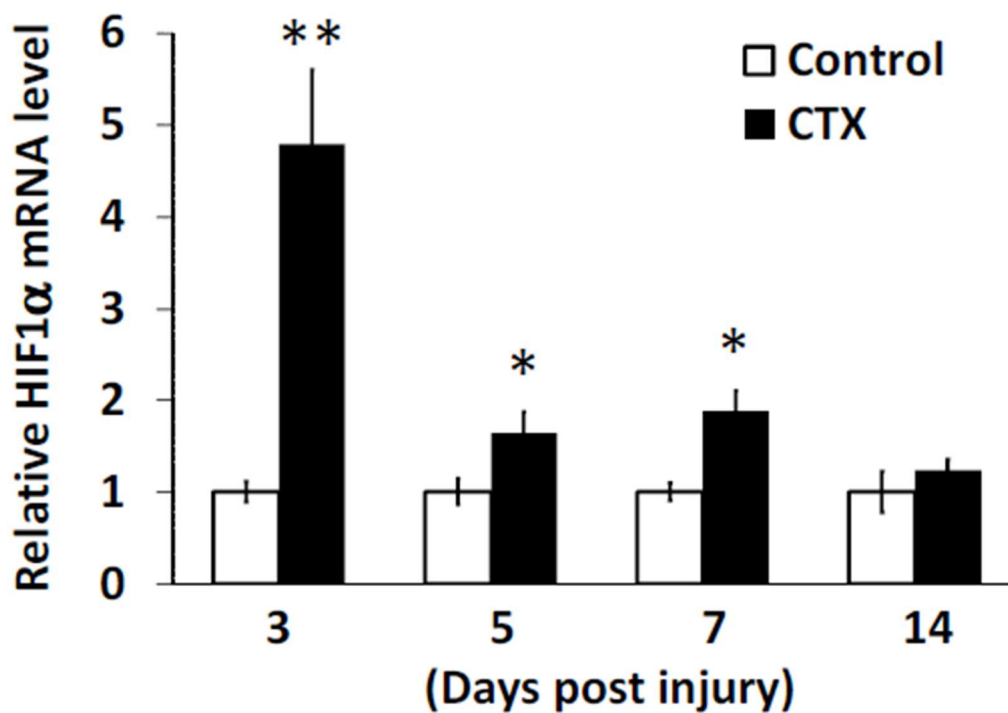
Figure 10. Relative mRNA level of HIF1α after injury.

Injury and control muscles were taken from 3 pairs of mice. HIF1α mRNA level significantly increased after injury and returned to normal condition by 14 days post CTX injection. Paired t test were used for statistical analysis. *p<0.05, **p<0.01)
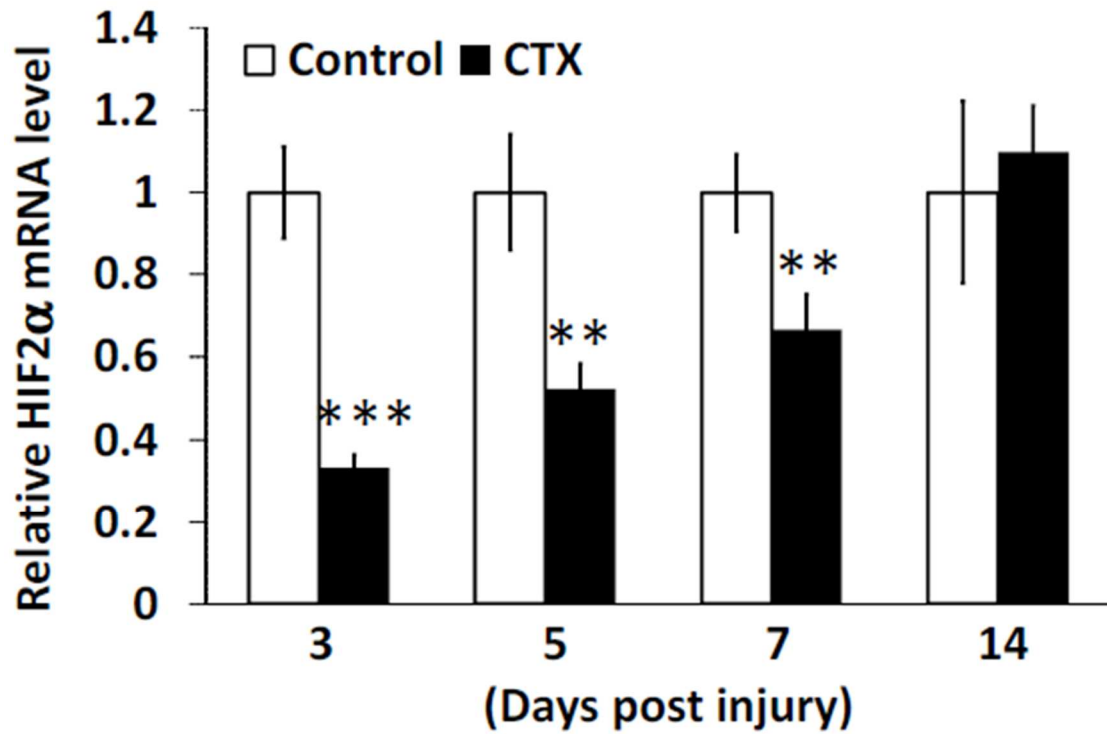
Figure 11. Relative mRNA level of HIF2α after injury.

After injury, HIF2α mRNA was significantly decreased and return to normal level during regeneration. (Injury and control muscles were taken from 3 mice as paired. Paired t test were used for statistical analysis. ***p<0.005, **p<0.01) (Figures are provided by Xin Yang)
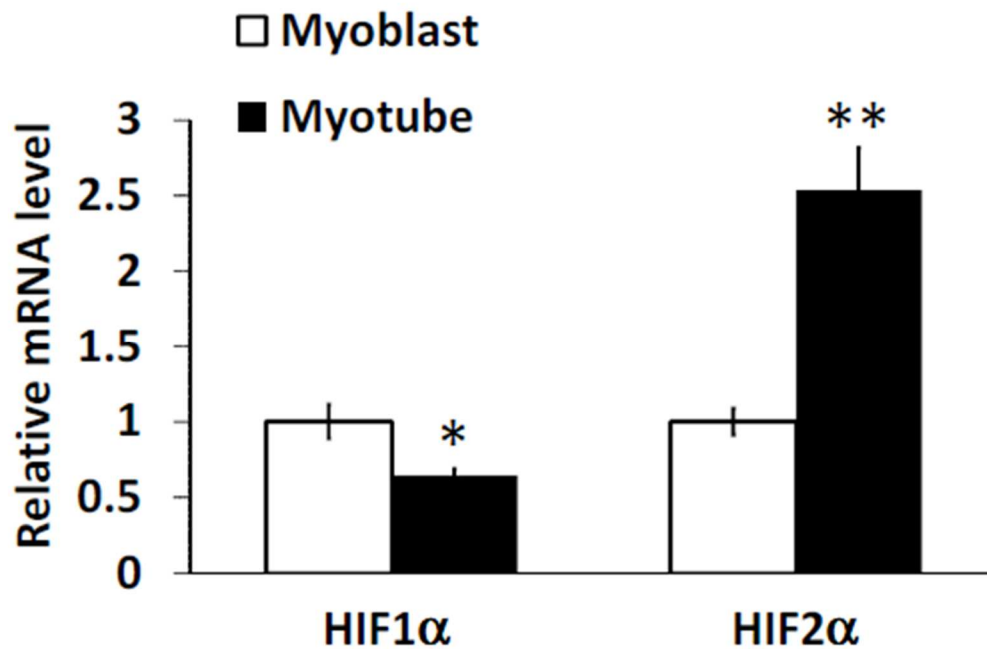
Figure 12. Different expression level pattern of HIF1α HIF2α in myoblast and myotube.

Samples were collected from 3 pairs of myoblast and myotube HIF1α levels were greater in myoblasts compared to myotube, while HIF2α level was higher in myotube versus myoblast. (paired t test. *p<0.05, **p<0.01)
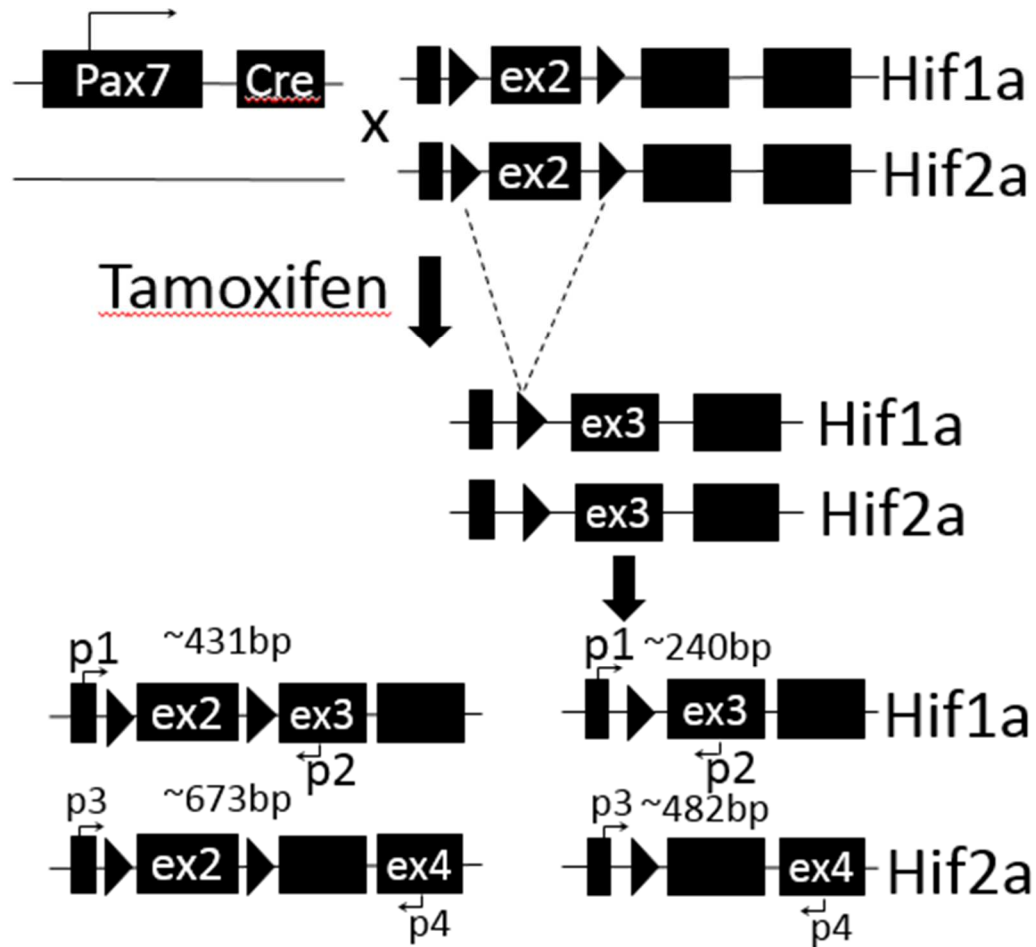
Figure 13. Breeding and validation strategy of Pax7 CreER double Knock out mice.
HIF1/2α$^{flox/flox}$ mice cross with Pax7 CreER mice to get Pax7CreER HIF1/2α$^{flox/flox}$ mice.
After Tamoxifen injection, Cre cleaves the second exon of HIF1α and HIF2α. Primers
were designed to the first and third exon to determine knock out efficiency.
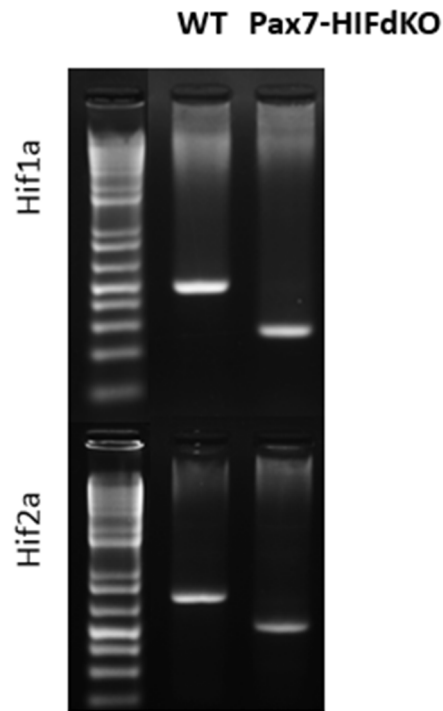
Figure 14. Validation of HIF1/2α knocking out in isolated myoblast.
Total mRNA extracted from control mice and tamoxifen post injected mice was reverse transcribed into cDNA. Target pieces of cDNA were amplified by designed primers. In control group, the amplified pieces were around 430 base pair for HIF1α and 670 base pair for HIF2α. While in Pax7-HIF^{dKO} group, the amplified pieces were around 240 base pair for HIF1α and 480 base pair for HIF2α. (Figures are provided by Xin Yang)
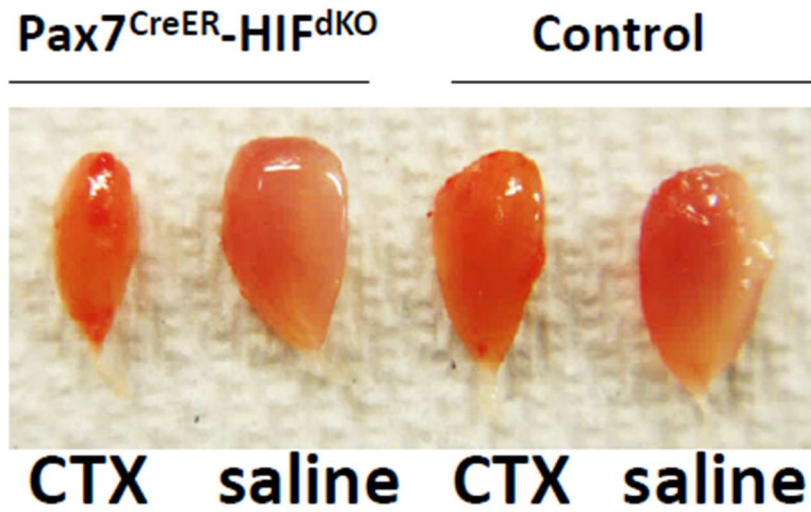
Figure 15. Representative picture of regenerated muscle 7 days post injury.
Pax7CerER-HIF^{dKO} mice showed poorer regeneration than control mice as CTX injured
muscle was significantly smaller in size in HIF-dKD than saline treated and control.
(Figures are provided by Xin Yang)
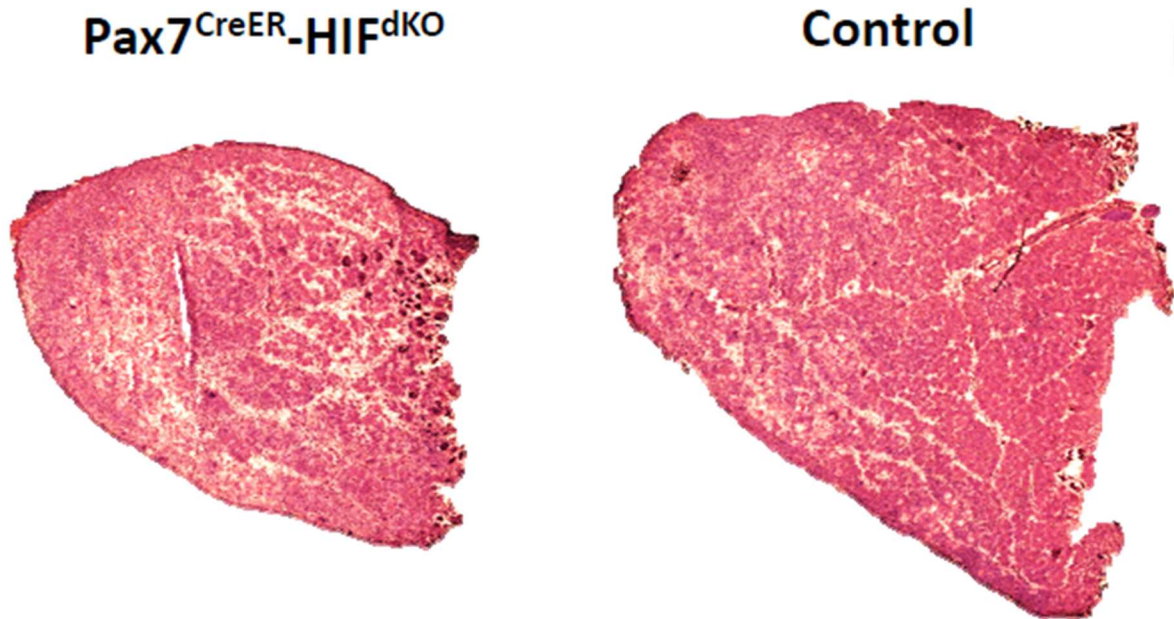
Figure 16. Representative picture of H&E stained regenerated muscle cross-section in low magnification (20X).

Pax7CerER-HIF^{dKO} mice exhibit more non-regenerated area and smaller cross-section area than control mice. (Figures are provided by Xin Yang)
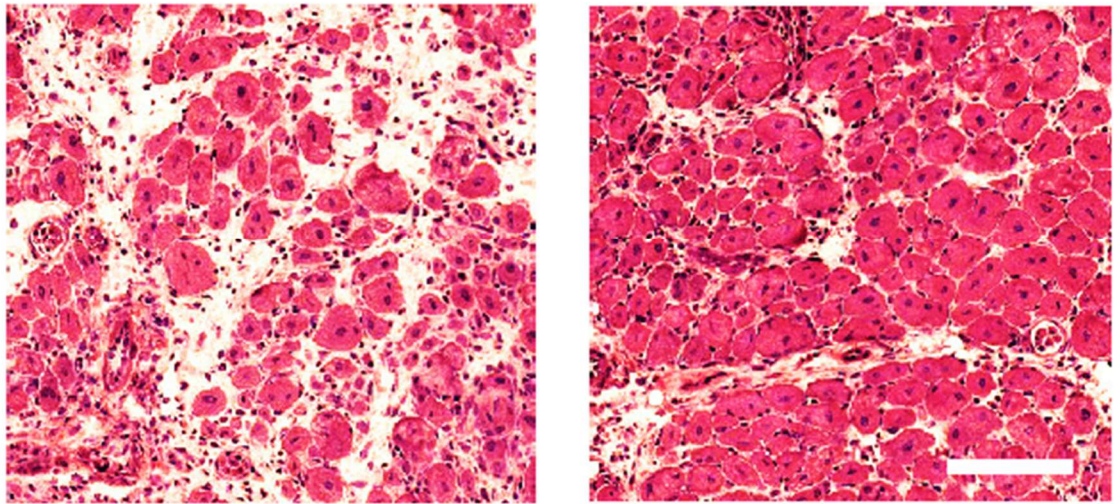
Pax7CreER-HIF^{dKO}   Control

Figure 17. Representative picture of H&E stained regenerated muscle cross-section at high magnification (200X).

Pax7CerER-HIF^{dKO} mice exhibit more non-regenerated area and smaller cross-section area than control mice. (Scale bar: 25 μm) (Figures are provided by Xin Yang)
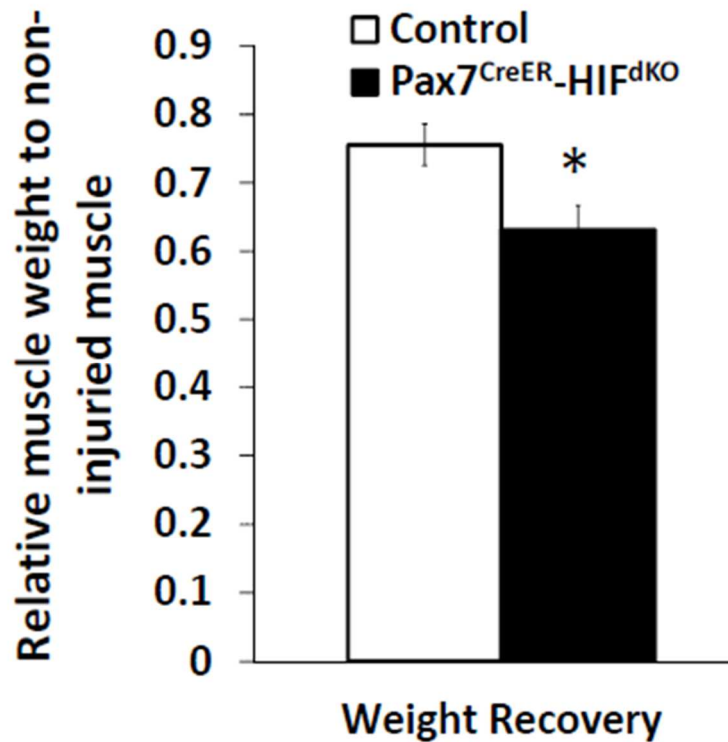
Figure 18. Impact of Pax7CreER-HIF^{dKO} on muscle weight after 7 days of regeneration.

The weight of regenerated TA muscle was measured 7 days post injury. Muscle weight recovery rate was calculated by regenerated muscle divide by non-injured muscle. Compared to control muscle, Pax7CreER-HIF^{dKO} mice exhibit lower muscle weight recovery rate. (Samples collected from 5 control and 4 Pax7CreER-HIF^{dKO} mice were measured for student t test. *p<0.05) (Figures are provided by Xin Yang)
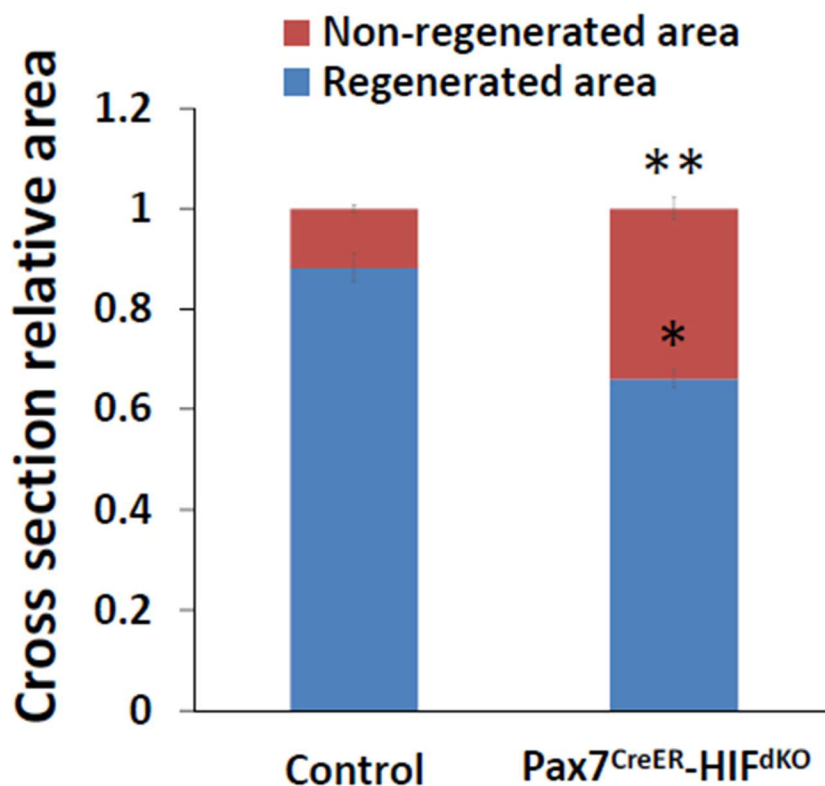
Figure 19. Measurement of regenerated area and non-regenerated area.
The regenerated area and non-regenerated area was measured from TA muscle cross-section area. Significant decreased regenerated area was noticed in Pax7CreER-HIF^{dKO} mice compare to control mice. (Samples collected from 5 control and 4 Pax7CreER-HIF^{dKO} mice were measured for student t test. *p<0.05, **p<0.01)
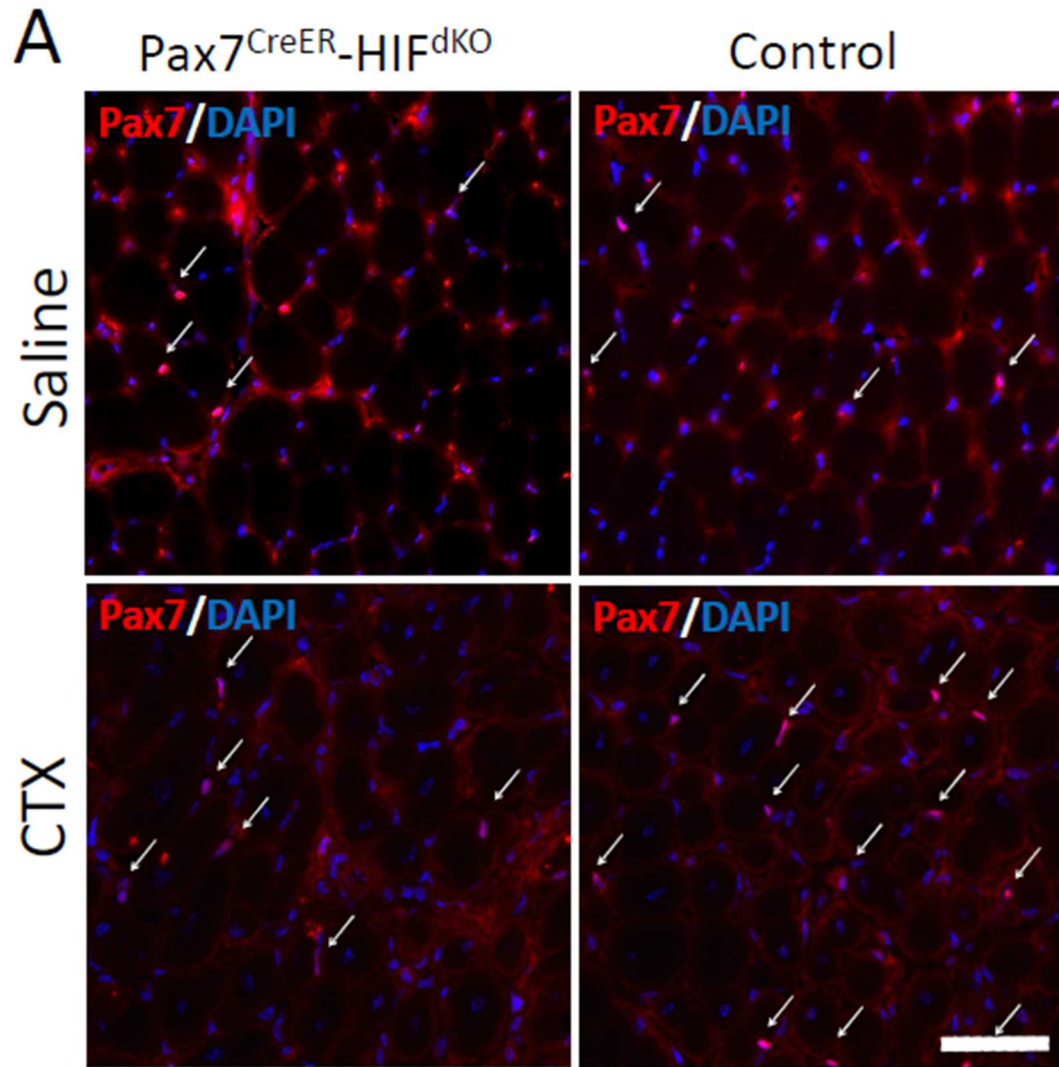
Figure 20. Representative picture of Pax7 staining of control and regenerated muscle cross-section in low magnification.

Pax7CerER-HIF^{dKO} mice exhibit less satellite cells than control mice. (scale bar: 25 μm)
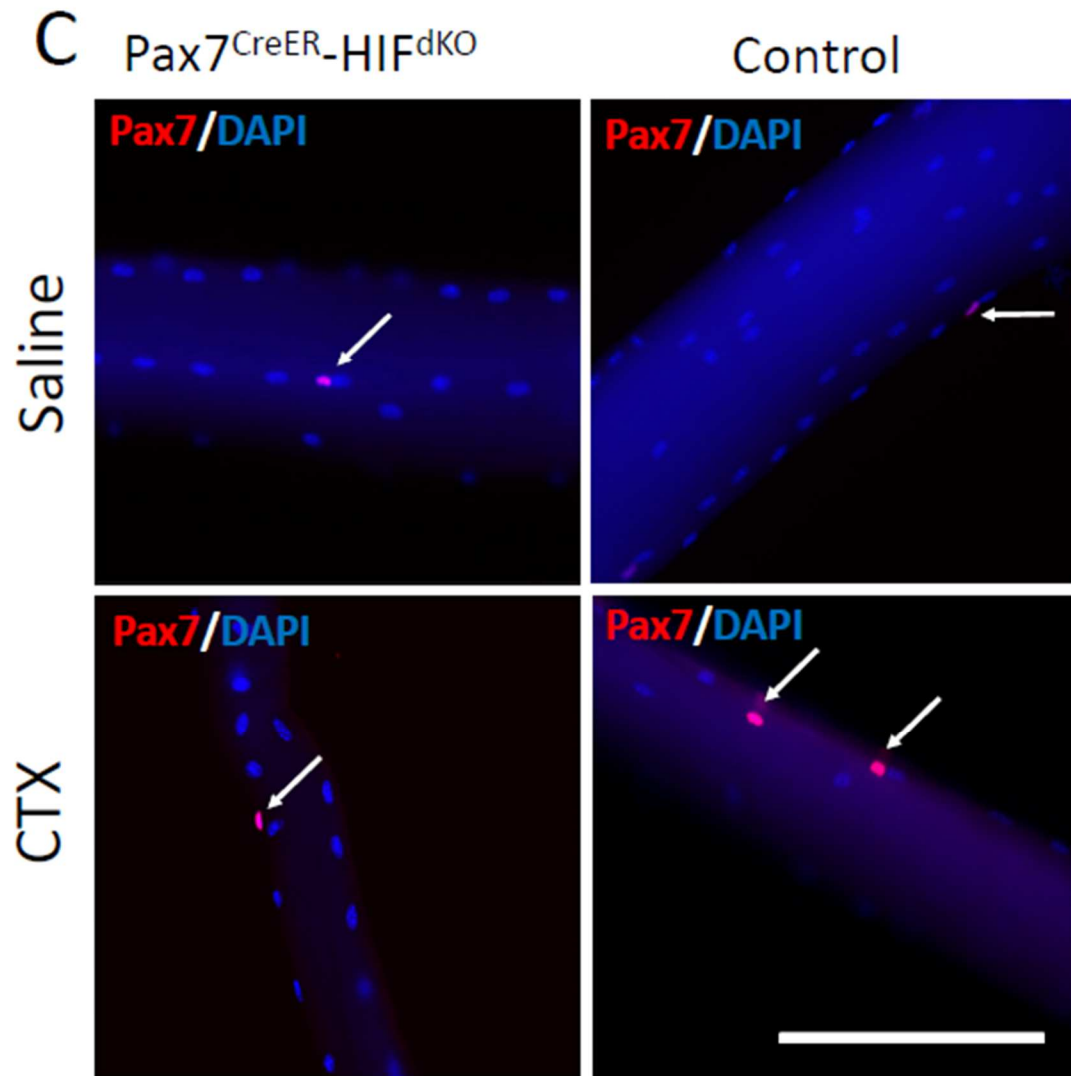
Figure 21. Representative picture of Pax7 staining of control and regenerated myofibers.

Pax7CerER-HIF^{dKO} mice exhibit less satellite cells than control mice 7 days post injury. (Scale bar: 25µm)
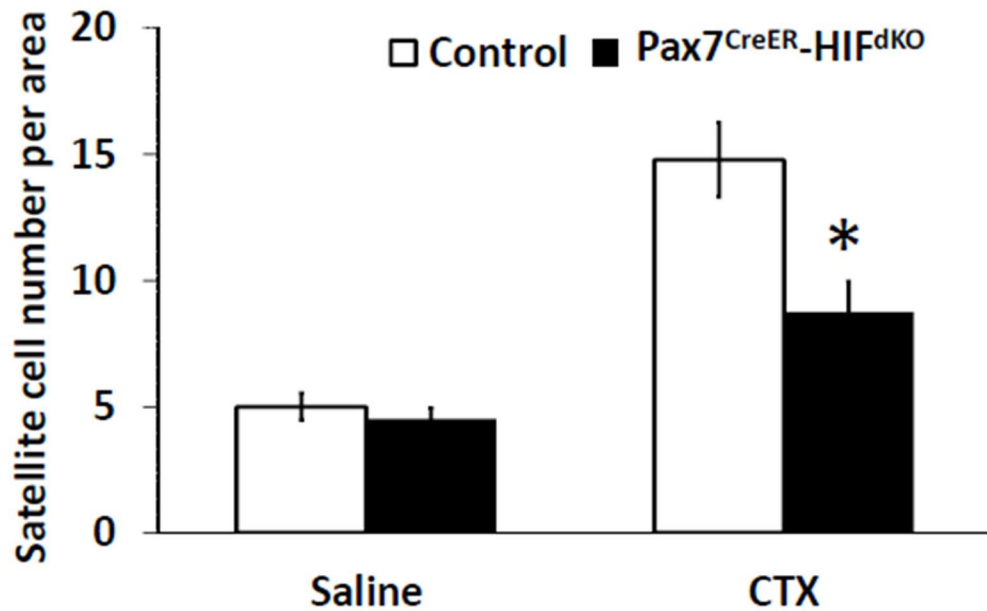
Figure 22. Quantification of satellite cells number from TA cross-section pictures.
Satellite cells were quantified by Pax7 immunofluorescent staining. Pax7 positive cells
are considered as muscle satellite cells. Pax7CerER-HIF^{dKO} mice exhibit significantly
reduced number of satellite cells 7 days post injury. (Samples collected from 5 control
and 4 Pax7CreER-HIF^{dKO} mice were measured for student t test. *p<0.05)
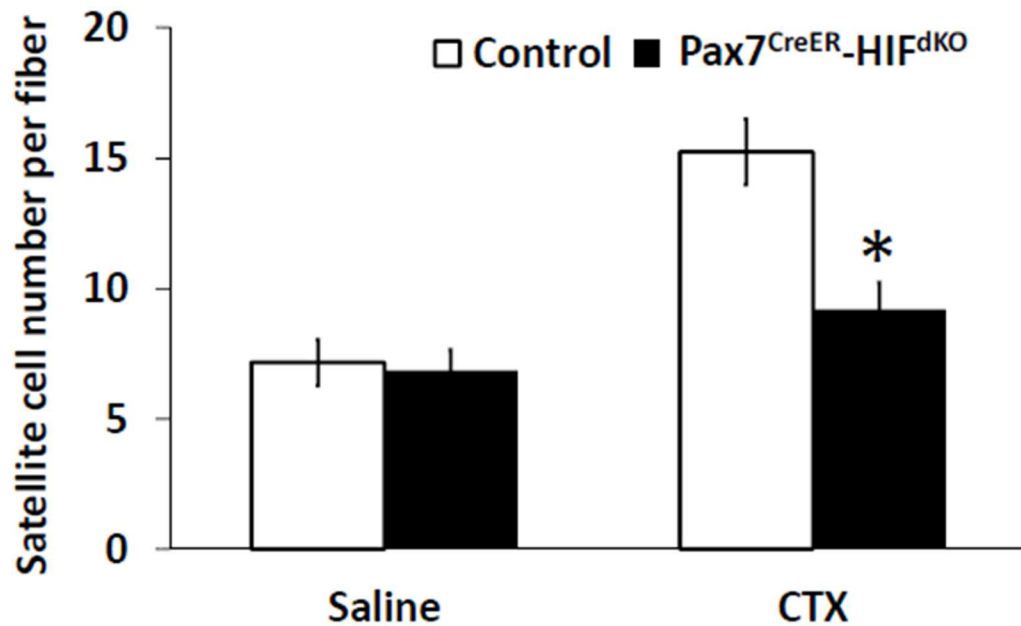
Figure 23. Quantification of satellite cells from myofiber Pax7 staining.
Satellite cells were quantified by Pax7 immunofluorescent staining. Pax7 positive cells are considered as muscle satellite cells. Pax7CerER-HIF^{dKO} mice exhibit significantly reduced number of satellite cells 7 days post injury. (Samples collected from 5 control and 4 Pax7CreER- HIF^{dKO} mice were measured for student t test. *p<0.05)
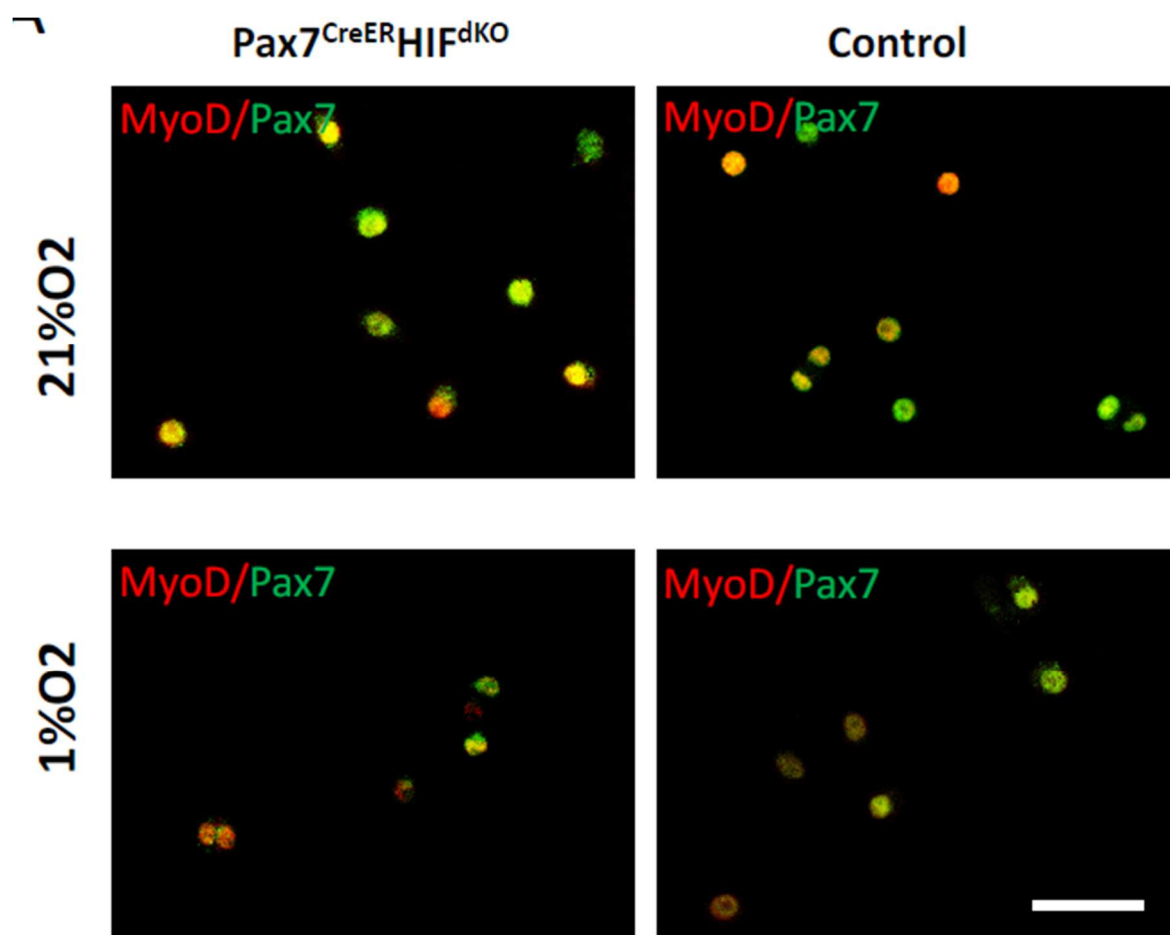
Figure 24. Represent pictures of MyoD/Pax7 staining for control and HIFdKO myoblast after cultured in normoxia and hypoxia for 72 hours.

Satellite cell cycle status were tested by MyoD/Pax7 staining. MyoD single positive cells are considered as differentiation, Pax7/MyoD double positive cells are classified as proliferation, while Pax7 single positive cells are classified as self-renewal. (Scale bar: 10 μm)
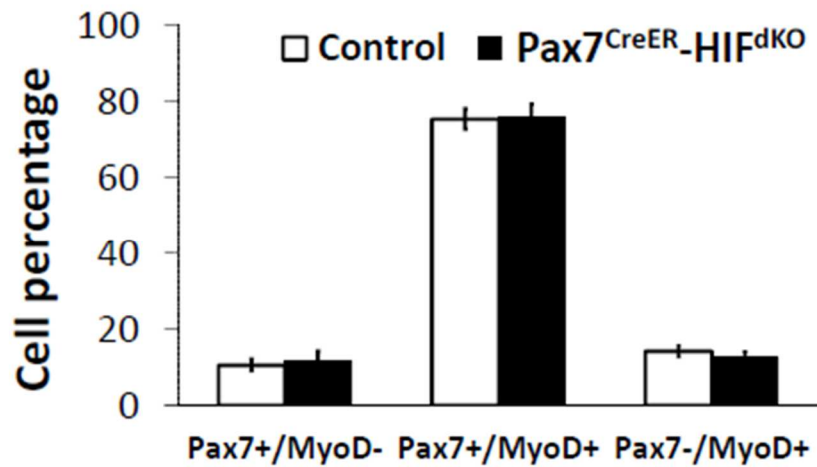
Figure 25. Quantification of Self-renewal, proliferation and differentiation from MyoD/Pax7 staining in normoxia.

Under normoxia condition, the self-renewal, proliferation and differentiation rate exhibited no significant difference between Pax7CreER- HIF^{dKO} and control mice. (Samples from 4 paired control and Pax7CreER- HIF^{dKO} myoblast were used for statistical analysis. Totally, around 500 cell numbers were counted for one sample.)
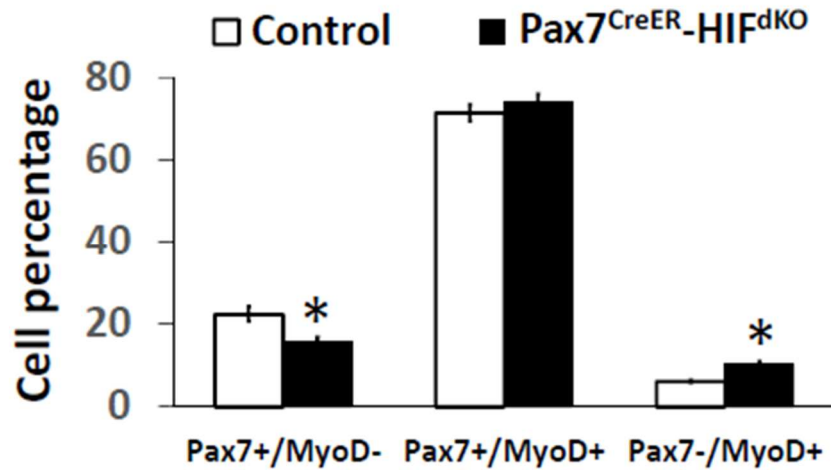
Figure 26. Quantification of Self-renewal, proliferation and differentiation from MyoD/Pax7 staining in hypoxia.

Under hypoxia condition, the self-renewal rate is lower while the differentiation rate is higher in Pax7CreER-HIF^{dKO} myoblast compared to control myoblasts. (Samples from 4 paired control and Pax7-HIF^{dKO} myoblast were used for statistical analysis. Totally, around 500 cell numbers were counted for one sample. *$p<0.05$)
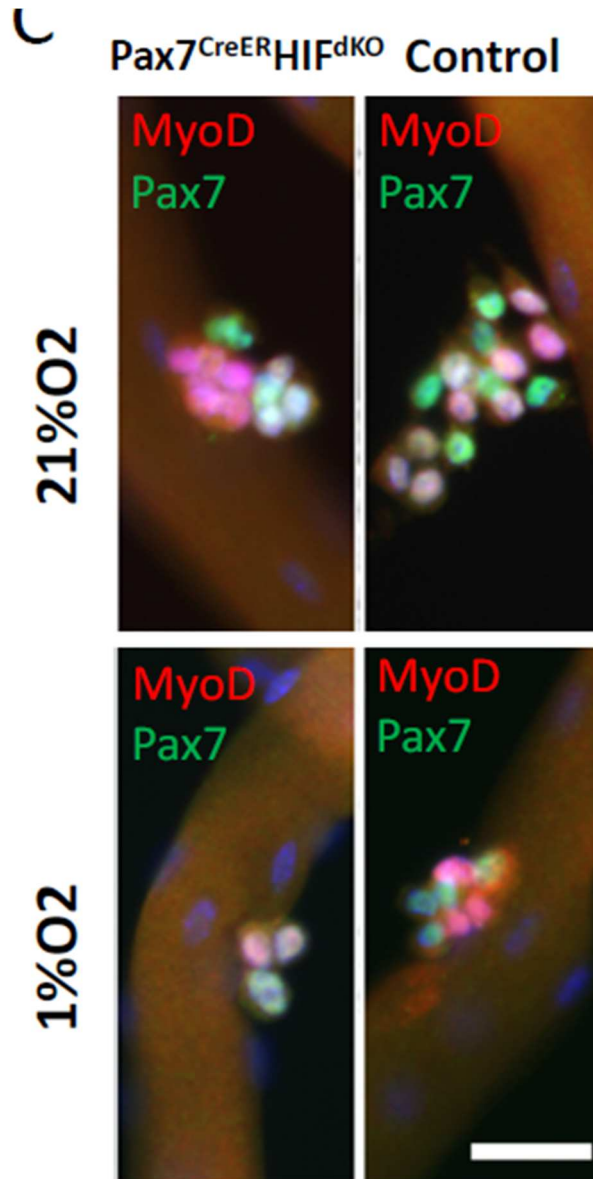
Figure 27. Represent picture of MyoD/Pax7 staining for control and HIFdKO myofiber after culture in normoxia or hypoxia for 72 hours.

To mimic in vivo microenvironment, we cultured myofiber in normoxia and hypoxia.

MyoD/Pax7 staining was used to test cell cycle stages as described before.
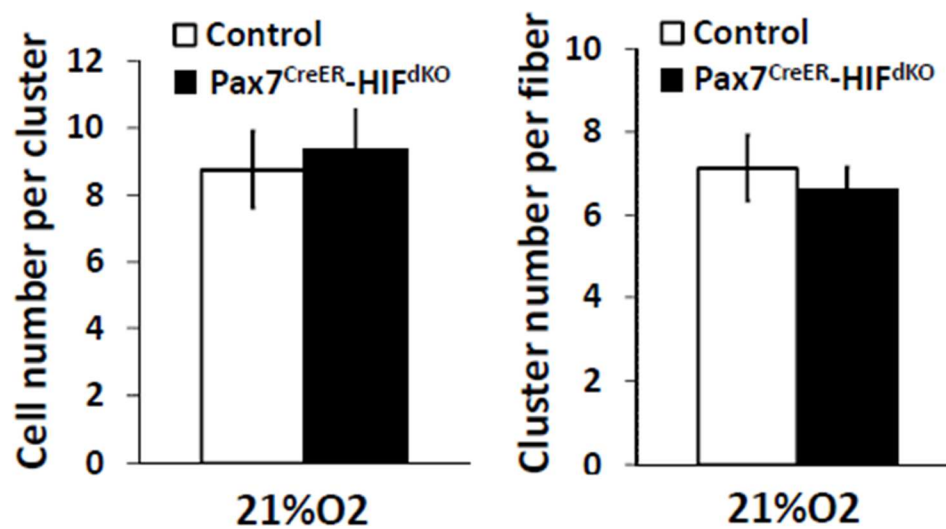
Figure 28. Quantification of cell number and cluster number after culture myofiber under 21%O2 level for 3 days.

No significant difference was noticed between Pax7CerER-HIF[dKO] and control mice under normoxia condition. (Samples from 4 paired control and Pax7-HIF[dKO] mice were used for statistical analysis. Totally, around 25 myofibers were counted for each sample.)

Figure 29. Quantification of cell cycle statues under normoxia condition after cultured for 3 days.

No significant difference was noticed between Pax7CerER-HIF^{dKO} and control mice under normoxia condition. (Samples from 4 paired control and Pax7-HIF^{dKO} mice were used for statistical analysis. Totally, around 25 myofibers were counted for each sample.)

Figure 30. Quantification of cell number and cluster number after 3 days cultured under hypoxia states.

Pax7CerER-HIF^{dKO} myofiber exhibited significant lower number of cluster and low number of cells per cluster. (Samples from 4 control and 5 Pax7-HIF^{dKO} mice were used for statistical analysis. Totally, around 25 myofibers were counted for each sample. *p<0.05)
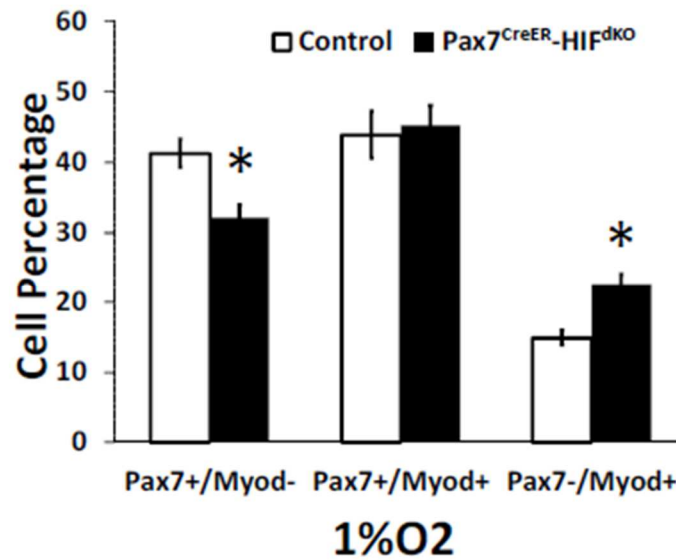
Figure 31. Quantification of cell cycle statues after culturing isolated myofibers under hypoxia states for 3 days.

Compared to myofibers isolated from control mice, myofibers from Pax7CreER-HIFdKO mice showed significantly lower self-renewal ratio and higher differentiation ratio. (Samples from 4 control and 5 Pax7-HIF^{dKO} mice were used for statistical analysis. Totally, around 25 myofibers were counted for each sample. *p<0.05)
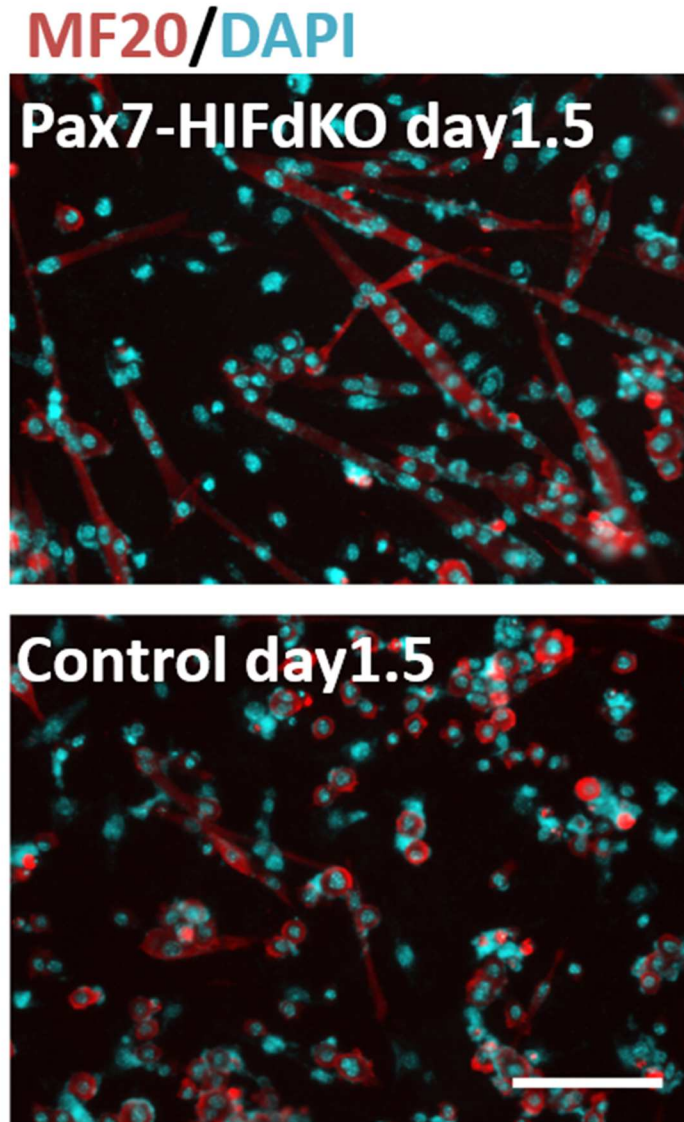
Figure 32. Representative picture of control and HIF^{dKO} primary myoblasts induced to differentiate in hypoxic conditions for 1.5 days.

Pax7-HIFdKO and control myoblasts were cultured in differentiation induction medium for 1.5 days under hypoxic conditions. To indicate myotube formation ratio, MF20 staining was used. HIFdKO myoblast exhibited higher differentiation ratio. (Scale bar: 10μm)
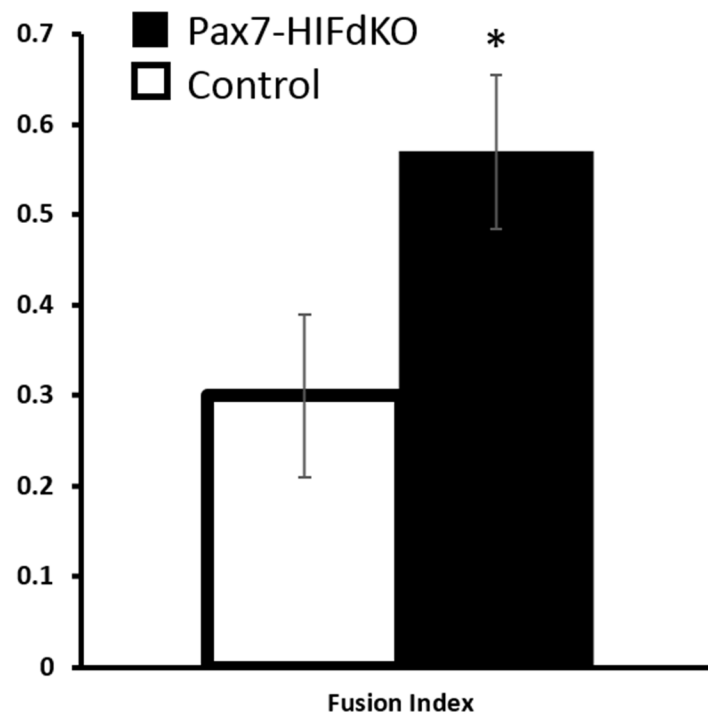
Figure 33. Quantification of differentiated myoblasts by fusion index.
Fusion index was calculated by divide total myoblast number over MF20 positive myoblast number. Pax7-HIF^{dKO} myoblast exhibited significant higher differentiated rate. (Samples from 4 paired control and Pax7-HIFdKO myoblast were used for statistical analysis, *p<0.05)
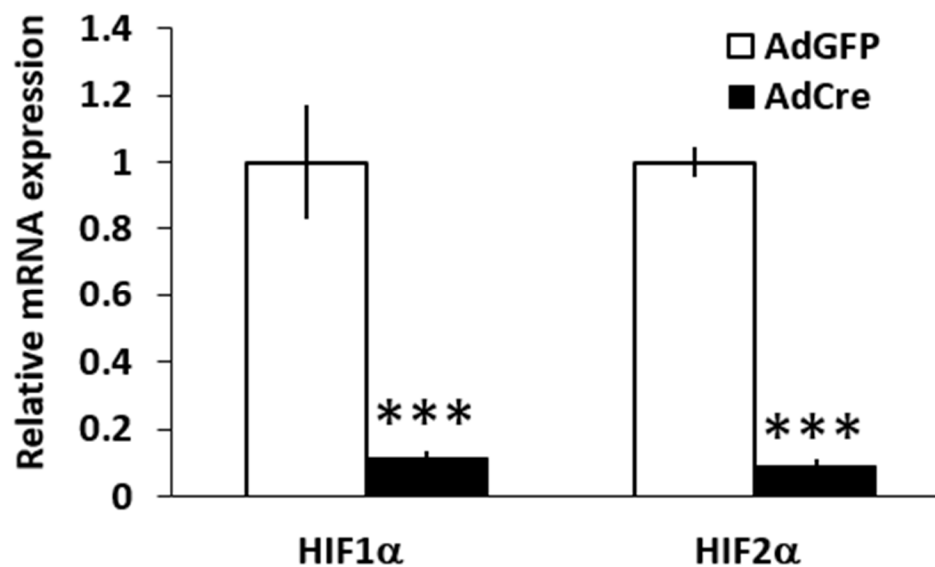
Figure 34. Relative mRNA of HIF1α and HIF2α after 24 transfection of adenovirus loaded Cre.

Total RNA was isolated from HIF1/2α double flox myoblasts transfected with adenovirus loaded Cre or adenovirus loaded GFP and q-PCR was used to measure relative expression of HIF1α and HIF2α (Samples were collected from 3 pairs of control and HIFdKO myoblast, student t test were used for statistical analysis. ***p<0.005)
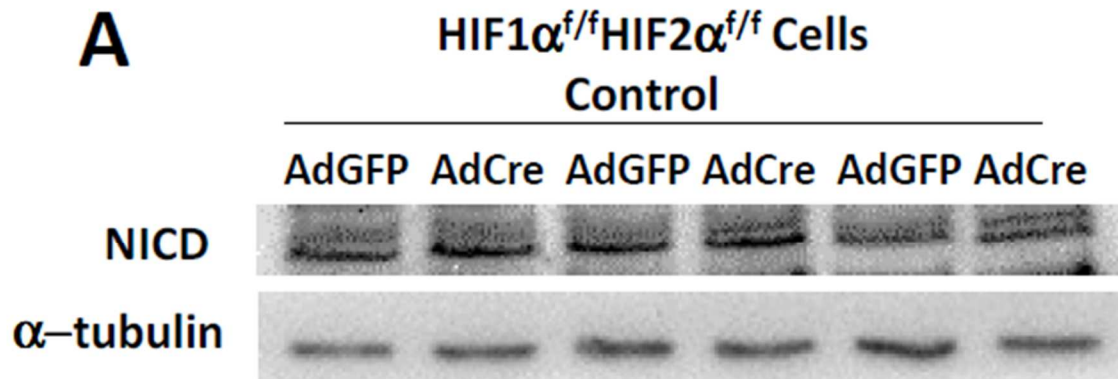
Figure 35. Western blot of NICD in control myoblast and HIFdKO myoblast after 48 hours culture under normoxia condition.

HIF1/2α double flox myoblast were treated by Adenovirus loaded Cre to induce knock out of HIF1/2α. Adenovirus loaded GFP was transfected into myoblast as control to diminish the side effect of transfection. After 48 hours culture, cellular protein was extracted and analyzed by western blot.
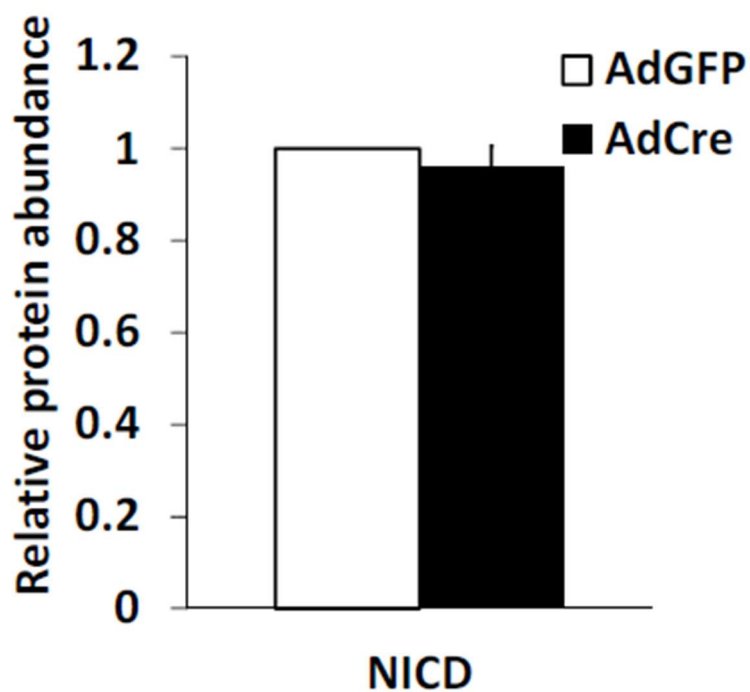
Figure 36. Quantification of NICD from Western blot of control myoblast and HIFdKO myoblast under normoxia condition.

Quantification of NICD level was conducted by using imageJ software by measuring the intensity of the represented blot band. There was no significant difference observed between control and HIFdKO myoblast. (N=3)
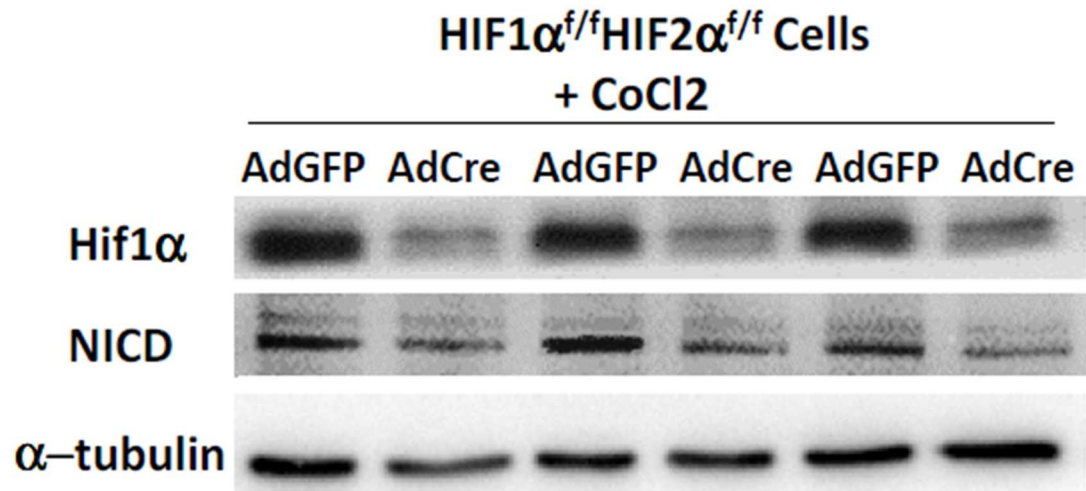
Figure 37. Western blot result of NICD and HIF1α from HIFdKO myoblast and control myoblast after 48 hours culture under hypoxia condition.
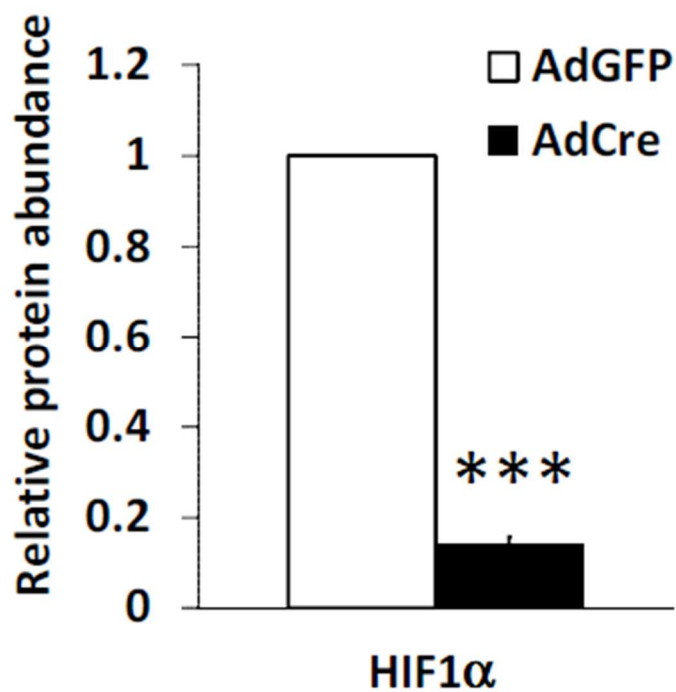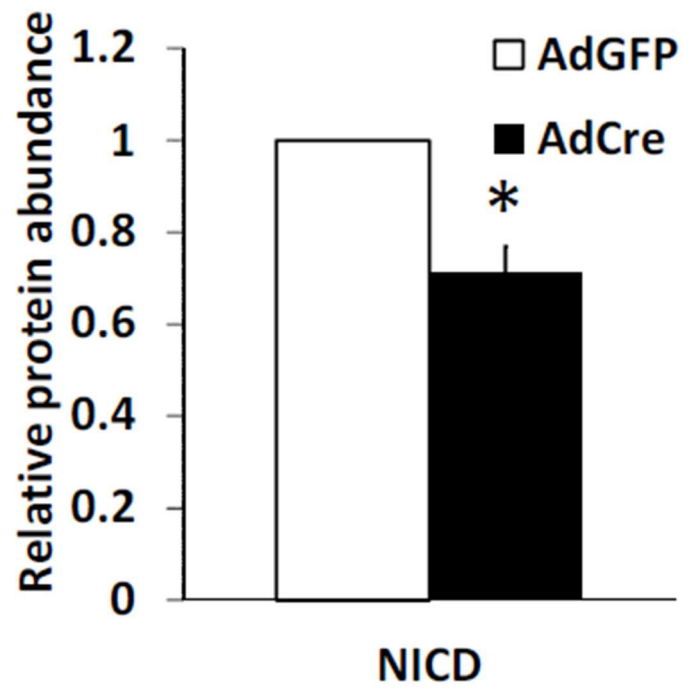
Figure 38. Quantification of HIF1α protein content.

HIF1a protein content was measured using intensity of represent bands in western blot. The HIF1α protein content was significantly lower in HIFdKO myoblast than in control myoblast (N=3, ***p<0.005).

Figure 39. Double knocking out HIF1α and HIF2α decrease NICD in hypoxic conditions.

NICD protein content was measured using intensity of represent bands in western blot. The NICD protein content was significantly lower in HIFdKO myoblast than in control myoblast. (N=3, *p<0.05)
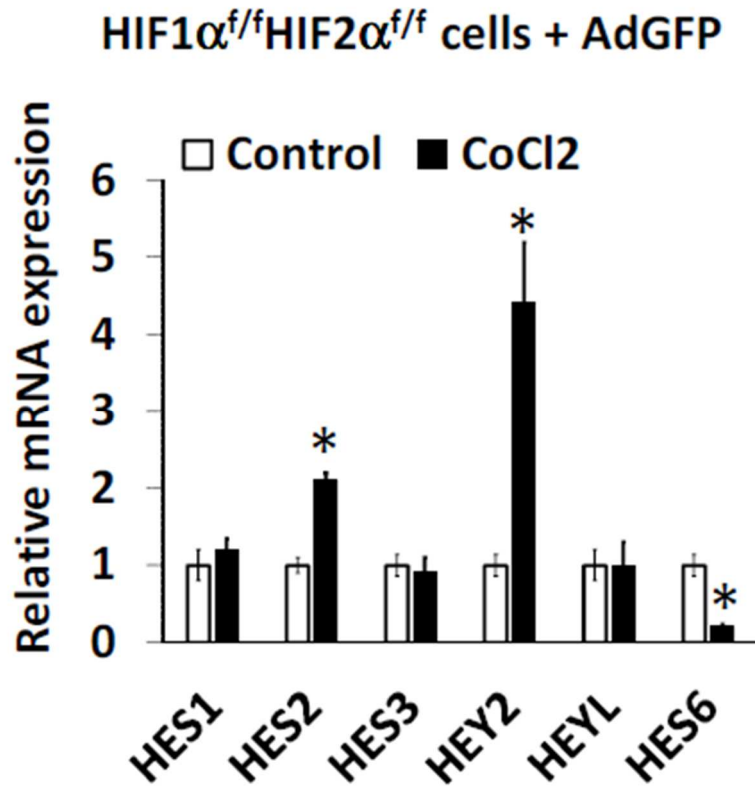
Figure 40. Notch target gene expression level changes before and after hypoxia treatment in control myoblast.

In control myoblasts, Notch activated genes were significantly upregulated after hypoxia treatment. (Samples from 5 paired control and HIFdKO myoblast were used for statistical analysis. *p<0.05)
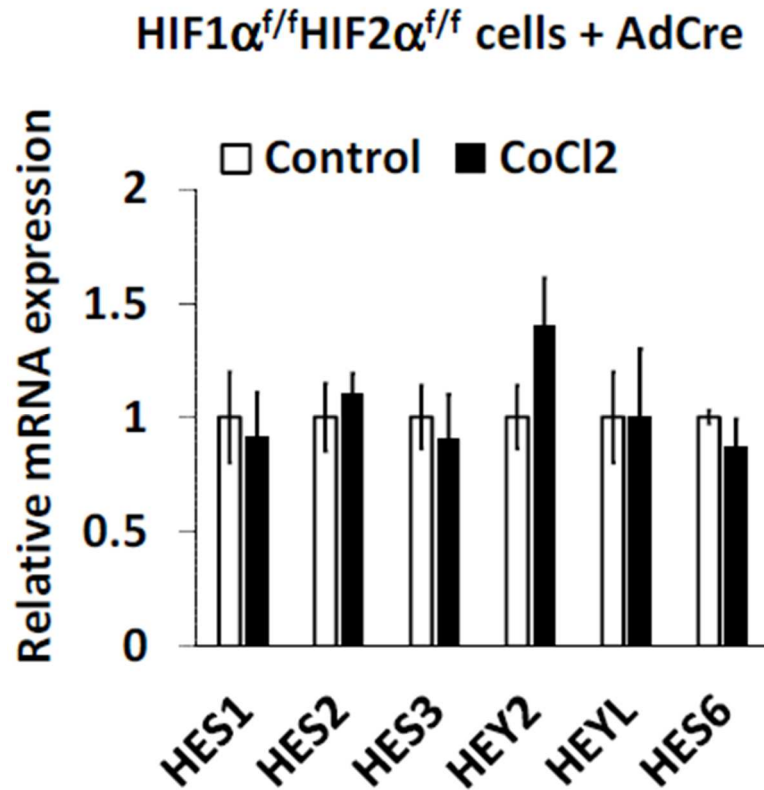
Figure 41. Notch target gene expression level changes before and after hypoxia treatment in HIFdKO myoblast.

In HIFdKO myoblasts, the promotion of Notch activated genes were significantly blocked after HIFdKO in hypoxia treatment. (Samples from 5 paired control and HIFdKO myoblast were used for statistical analysis. *p<0.05)
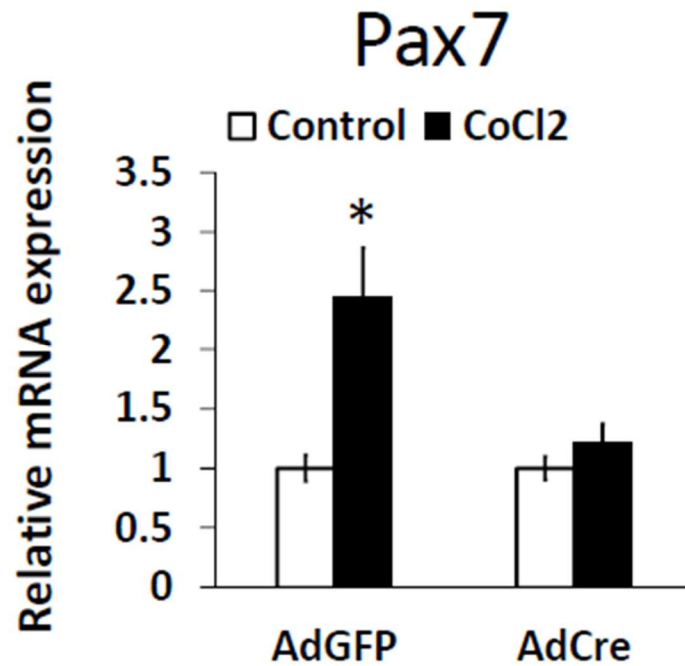
Figure 42. Pax7 mRNA expression in control and HIFdKO myoblast before and after 48 hours hypoxia treatment.

Pax7 expression level was significantly elevated in control myoblast after hypoxia treatment. While in HIFdKO myoblast, Pax7 expression changes not significantly after hypoxia treatment. (Samples from 5 paired control and HIFdKO myoblast were used for statistical analysis. *p<0.05)
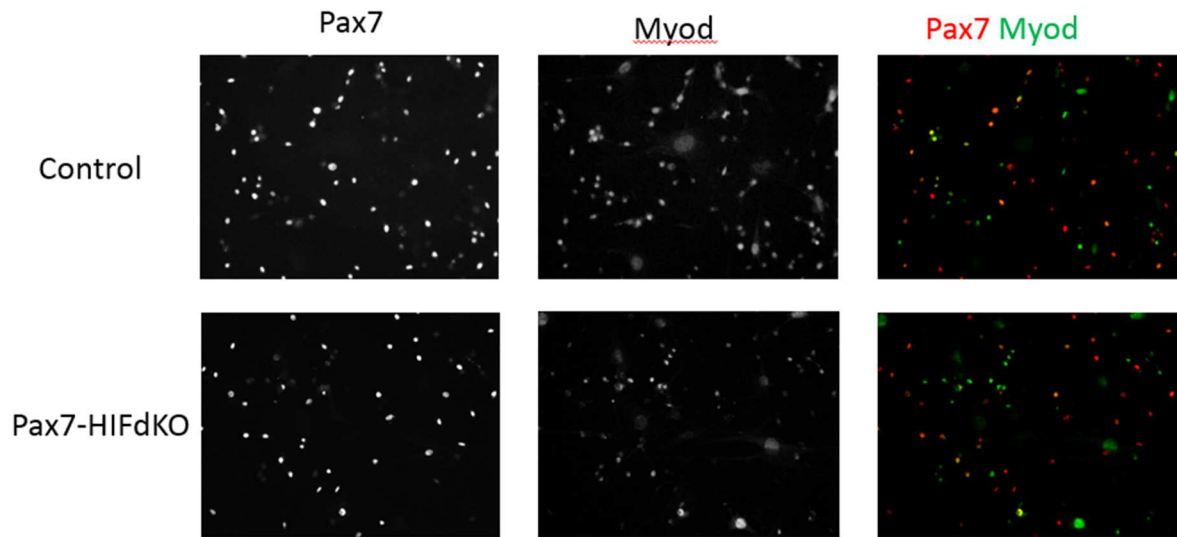
Figure 43. Represent picture of Pax7/Myod staining of OP9 co-cultured myoblasts.
OP9 cell lines were used for continuously activation of Notch for rescue HIFdKO.
Myoblasts were co-cultured with OP9 cells for two days for cell stage analysis.
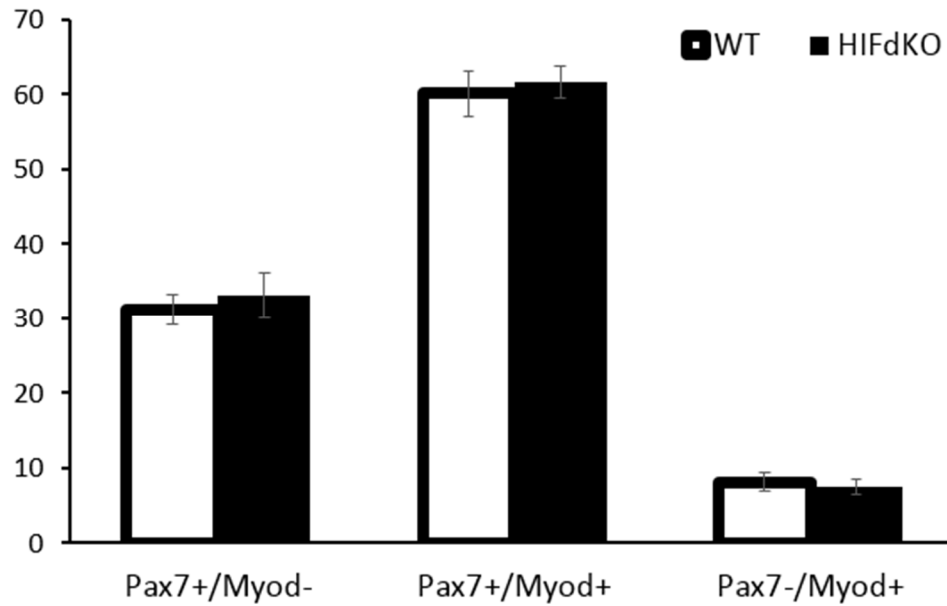
Figure 44. Quantification of cell stages for OP9 co-cultured myoblast.
There was no significant difference in cell states noticed between control and HIFdKO myoblast after continuously activation of Notch signaling. (Samples from 4 paired control and Pax7-HIFdKO myoblast were used for statistical analysis.)

VITA

Shiqi Yang is the child of Xinquan Yang and Xinjiao Chen. He is born in 1991 at Puyang, Henan, PRC. Shiqi graduated from Youtian No.1 High School in 2009. He obtained his Bachelor degree of Agronomy from China Agriculture University. After that he contained his study as a master student under the supervision of Dr. Shihuan Kuang at Purdue University in Department of Animal Sciences. Shiqi strived to be a highly motived individual who have broad interests in computation, biology and engineering. He hopes to use the knowledge he obtained to make agriculture move forward.