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Effects of hypoxia on proliferation and differentiation of myoblasts

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Summary Oxygen is an environmental and developmental signal regulator, and its role is involved in energy homeostasis, development and process of differentiation. Myoblasts persist in skeletal muscle as satellite cells, which possess capability of self-renewing and differentiation into mature myofiber. Myoblasts play a critical role in postnatal muscle regeneration after injury as well as maintaining myofibers' function. Though oxygen is vital to nearly all forms of life, studies focused on investigating the effects of oxygen level on proliferation and differentiation of myoblasts are few. Lower oxygen concentration is more close to the level of oxygen in physiological and pathological environment *in vivo*. So physiological environment is actually optimum condition for myogenesis. It is significant for understanding repair and regeneration of skeletal muscle to study on effects of hypoxia on myogenesis. HIF-1 signaling pathway was involved in these processes as well as other signaling pathways would be, and accordingly, deep studying and further revealing the signaling pathways involved in mechanism will provide evidences or references for looking for novel targets for stem cells therapy and drug treatment.

Introduction

Oxygen, which is vital to nearly all forms of life on earth, plays an important role in energy metabolization and homeostasis as well as a variety of life processes. Changes of oxygen concentration, as an important physiological and pathological regulator, might influence the whole life span, from embryogenesis and development to the maintenance of normal function, dysfunction, disease and aging. The concentration of oxygen in atmosphere is 21%, whereas the level of oxygen at the tissue *in vivo* is significantly less. Arterial blood is about 12% oxygen, and the mean tissue level of oxygen is about 3%, with considerable local and regional variation. These means are for adult organs and tissues. Mean oxygen tension at the tissue level in embryos is considerably less [1].

Many evidences have demonstrated that a few muscle stem cells exist in adult mammalian skeletal muscle. Muscle stem cells can be activated to initiate proliferation and give rise to spindly myoblasts, which undergo differentiation and cell fusion into multinucleated myotubes. It is obvious that the tissue-specific myoblasts are required for

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maintenance and repair of postnatal skeletal muscle. The partial saturation of oxygen found in mature skeletal muscle is reported to be between 1% and 10% [2,3], however, the atmospheric conditions used in traditional muscle cells culture *in vitro* have been largely ignored. Recent experiments have suggested that maintaining cells under more physiological atmospheric conditions may have many benefits [4]. So physiological environment is actually optimum condition for myogenesis. It is significant for understanding repair and regeneration of skeletal muscle to study on effects of hypoxia on myogenesis. Especially studies on effects of local and regional microenvironment hypoxia on myoblasts proliferation and differentiation have been hardly carried out up to now.

Regulation of myoblasts proliferation and differentiation

Skeletal muscle differentiation is characterized by myoblasts withdrawal from the cell cycle, induction of muscle-specific gene expression, and cells fusion into multinucleated myotubes. The antagonism between proliferation and differentiation implies that signaling pathways driving proliferation must be suppressed to allow induction of differentiation. The muscle regulatory transcription factors (MRFs) MyoD, myogenin, MRF4, and Myf5 were initially identified as master regulators of myogenic differentiation [5—8]. In addition to MRFs, several others such as Pax7 [9], AP-1 [10], NF-κB [11] and FOXO1a [12] were reported to be associated with myogenesis. Proliferating myoblasts express MyoD and Myf5 before the onset of muscle differentiation. Once differentiation activated, MyoD and Myf5 induce the withdrawal of myoblasts from the cell cycle together with the expression of myogenin. Myogenin and MRF4 possess functions that regulate myoblasts terminal differentiation further into myotubes. The link between myoblast cell cycle withdrawal and differentiation is established through regulation between cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CDKIs). The CDKI p21 and the retinoblastoma protein (Rb) are critical in establishing the post-mitotic state during myogenesis [13,14]. In myogenic cells, p21 expression increases during the G_1 phase of the cell cycle, and this up-regulation is associated with permanent cell cycle arrest [15]. One key target of the CDKs is Rb, which is regulated by its phosphorylation state. p21, by blocking CDK activity, induces accumulation of Rb in the hypophosphorylated, active form, which is necessary for the maintenance of the permanent cell cycle withdrawal in myotubes [13,16,17]. MyoD is a key transcription factor regulating both p21 and Rb gene expression during muscle differentiation. MyoD has been found to enhance activity of the p21 promoter in transient transfection experiments [15] and to stimulate p21 mRNA and protein accumulation in muscle cells and fibroblasts [15,16].

In addition to MyoD family of bHLH factors MRFs, many growth factors are involved in regulating proliferation and differentiation of myoblasts. However, only a few among these growth factors' regulation mechanisms were clarified and others further investigations have been undergone. At present, some recognized growth factors via their roles in regulating proliferation and differentiation of myoblasts are insulin-like growth factor-I/-II (IGF-I, IGF-II), hepatocyte growth factor (HGF), fibroblast growth factors (FGFs), transforming growth factors (TGFs), interleukin-6 cytokines (IL-6) family and so on. Different growth factors via different signaling pathways inhibit or induce determinate differentiation of myoblasts. Chen et al. [18] investigated the direct effect of CNTF on skeletal myoblasts of adult human. they found that CNTF induced the myogenic lineage-committed myoblasts at a clonal level to dedifferentiate into multipotent progenitor cells—they not only could proliferate for over 20 passages with the expression absence of myogenic specific factors Myf5 and MyoD, but they were also capable of differentiating into new phenotypes, mainly neurons, glial cells, smooth muscle cells, and adipocytes. These ''progenitor cells'' retained their myogenic memory and were capable of redifferentiating into myotubes. Furthermore, CNTF could activate the p44/p42 MAPK and down-regulate the expression of MRFs.

Effects of hypoxia on proliferation and differentiation of myoblasts

Since the level of oxygen *in vivo* is more less than the atmospheric conditions (21% O_2) used during traditional cell cultivation *in vitro*. The low oxygen levels are actually ''normoxic'' for the time and place in development. This physiological hypoxia condition which was required for cells and tissues has been largely ignored by most researchers. Recently, there has been a little attention given gradually to the role of local microenvironment hypoxia in development. Some experiments were performed in the low oxygen level to mimic the internal physiological or pathological conditions and suggested that maintaining cells under more

physiologic environment may be better to understand the processes of cell proliferation and differentiation as well as how to maintain the functions of tissues and organs. The partial saturation of oxygen found in mature skeletal muscle is reported to be between 1% and 10%. The level of oxygen is more less than this in skeletal muscle which were exercised or damaged. In 2000, Morrison et al. [19] and Studer et al. [20] first reported that central nervous system and neural crest stem cells had increased rates of survival and proliferation, reduced apoptosis, and increased dopamine neurons generation when cultured in a decreased oxygen environment. Based on these results, Chakravarthy et al. [21] for the first time examined the effects of decreased oxygen on the activation and proliferation of satellite cells from aged rats. They cultured satellite cells isolated from 31-month-old rats under standard culture conditions with 21% O_2 and under atmospheric conditions containing approximately 3% O_2. they noted that primary muscle cells cultured in 3% O_2 had a significant increase in proliferation rate and formed larger myotubes than control cells cultured in 21% O_2. Proliferating myoblasts cultured in lowered oxygen had increased protein levels of G_1/S cyclins and cyclin-dependent kinases, as well as decreased levels of the cell cycle inhibitor p27. Akt phosphorylation was significantly enhanced after the 3rd day of low oxygen. Actived Akt, phosphor-Akt, is one of the central regulators of cellular proliferation, especially via its interaction with several of the G_1/S cell cycle molecules.

The results found in the experiments of Csete et al. [22] were similar to Chakravarthy's results. They examined the effects of O_2 levels on the developmental potential, proliferative capacity, and phenotype of the adult skeletal muscle fiber progenitor population (satellite cells) and cell lines that model multipotential embryonic paraxial mesoderm from which skeletal muscle developed. They found that both satellite cells proliferation and survival of mature fibers were enhanced in 6% O_2, and 6% O_2 conditions also accelerated the upregulation of multiple MyoD family myogenic regulatory factors. Physiologic levels of O_2 in culture decreased satellite cells adipogenesis and promoted myogenesis. These results suggested that signaling pathways involving in regulating proliferation and differentiation phenotype of stem cells are impacted by the surrounding O_2 microenvironment where cells exist. Our laboratory's work with rat myoblasts also indicated that the number of myoblasts cultured in 3% and 10% O_2 conditions increased by 1.5 and 2.5 times compared with control group, respectively [23].

Recently, Di Carlo et al. [24] investigated the effect of hypoxia on myogenic differentiation of C2C12 myoblasts. They observed that $\sim$1% O_2 negatively regulated myogenic differentiation by inhibiting MRFs expression and cell cycle withdrawl. Hypoxia significantly impaired C2C12 proliferation in GM. Proliferating C2C12 myoblasts were cultured in DM for different time periods up to 72 h either under normoxic or hypoxic conditions. They noted that C2C12 cultured in normal oxygen levels fused into myotubes and MyHC expression increased over time, in contrast, C2C12 cultured in hypoxia for different time periods showed neither myotube formation nor MyHC protein accumulation, which indicated that hypoxia inhibited myogenic differentiation of myoblasts. To analyze the myogenic differentiation inhibition by hypoxia was whether temporary or permanent, C2C12 myoblasts were placed in DM in hypoxic conditions for 48 h and then shifted to normoxic conditions either in GM or in DM. When shifted to normoxic GM, C2C12 entered the cell cycle, proliferated, and did not express MyHC. When shifted to normoxic DM, C2C12 underwent myotube formation and MyHC accumulation. These results indicated that myoblasts differentiation was reversibly inhibited by hypoxia, as myoblasts retain their capacity to proliferate or differentiate when normal oxygen levels were restored. They sought to analyze the effect of hypoxia on the cell cycle profile of C2C12 myoblasts and observed that hypoxic myoblasts were reversibly arrested in G_1. Carlo et al. demonstrated that hypoxia inhibited myogenic differentiation through accelerated MyoD degradation by the ubiquitin-proteasome pathway. Hypoxia by inducing MyoD degradation blocked accumulation of early myogenic differentiation markers such as myogenin and p21 and Rb, and inhibited myoblasts differentiation.

Yun et al. [25] investigated the effects of three different levels of hypoxia on myogenesis. These three oxygen levels were respectively physiological hypoxia at 2% O_2, pathological hypoxia at 0.5% O_2, and extreme pathological hypoxia at 0.1% O_2. Their studies indicated that myogenesis was inhibited at $\leqslant$2% O_2 and the strongest inhibition was found at 0.01% O_2. When C2C12 cells were transferred to 21% O_2 after the cells induced myogenic differentiation at 2%, 0.5% or 0.01% O_2 for 3 days, they recovered to continue differentiation from 2% O_2 and 0.5% O_2, and but only to some extent from 0.01% O_2. They also investigated the adaptive myogenic differentiation of C2C12 myoblasts by continuous exposure of differentiating myoblasts to 0.5% O_2 for up to 12 days. They found that myogenesis was significantly repressed on day 3, extensive

myogenesis occurred by day 6 and progressed further by day 12 at 0.5% O_2. These findings indicated that myogenesis was able to adapt to chronic hypoxia. This adaptive mechanism was accompanied by initial inhibition of the myoD, E2A, myogenin genes followed by resumption of their expression in an oxygen dependent manner. The transcription of myoD gene was regulated by hypoxia which inhibited transiently deacetylation of histones associated with the myoD promoter and repressed myoD mRNA. However, these results were inconsistent with Carlo's results that hypoxia accelerated MyoD degradation. It is needed to be investigated further that hypoxia influencing the expression of MyoD should occur whether at transcription level or post-transcription level.

The role of HIF-1 during myogenesis of myoblasts

HIF-1 is an important transcription factor expressed in response to hypoxic conditions. At a molecular, HIF-1 is a heterodimer composed of a oxygen-sensitive HIF-1α subunit and a constitutive-expressed HIF-1β subunit which is identical to the aryl hydrocarbon receptor nuclear translocator (ARNT). Both of them are members of the basic helix-loop-helix (bHLH)-PAS family. Some researches have demonstrated that the expression of HIF-1α protein had been changed in response to hypoxia but the level of HIF-1α mRNA had remained unchanged under hypoxic conditions. Under normoxia, HIF-1α protein is expressed but is unstable and undergoes continuous proteolysis through the ubiquitin-proteasome pathway. HIF-1α degradation requires binding of VHL (the product of the Von Hippel Lindau tumour suppressor gene) which acts as the recognition component of an E3 ubiquitin ligase complex. VHL binds exclusively HIF-1α modified by praline hydroxylases in the oxygen dependent degradation domain (ODD) within the HIF-1α protein. These enzymes are oxygen dependent and HIF-1α cannot be hydroxylated during hypoxia. The HIF-1α—VHL interaction strictly requires hydroxylation, and therefore HIF-1α accumulates and translocates into the nucleus, followed by binding to HIF-1β to form the active transcription factor HIF-1 during hypoxia [26]. However, the mechanisms of HIF-1α stabilization might take on diversity based on different cell phenotypes or tissues. The effects of hypoxia on HIF-1α in muscle cells are different than do in other tissues. For non-muscle phenotype cells, hypoxia inhibits HIF-1α degradation via the ubiquitin-proteasome pathway and results in increasing expression of HIF-1α. For myoblasts, hypoxia promotes nuclear import of HIF-1α and does not inhibit significantly proteasomal degradation. Under hypoxia, the binding of HSP90 to HIF-1α was weaker in myocytes [27]. Stroke et al.' research shown that skeletal muscle tissue contained remarkable amounts of HIF-1α under normoxia as well as under hypoxia compared with other tissues [28]. Kubis et al. [27] investigated HIF-1α accumulation and subcellular localization in primary skeletal muscle cells exposed to low oxygen concentration (3% O_2), normoxia (20% O_2) or high oxygen concentration (42% O_2). They cultured rabbit primary myoblasts and found that significant amounts of HIF-1α located exclusively in the cytoplasm of the myotubes under normoxic and high oxygen conditions with only a minor reduction compared with did under low oxygen concentration. Muscle cells treated with $CoCl_2$ which can mimic hypoxia, a known inhibitor of HIF-1α degradation, shown even higher levels of HIF-1α and again exclusively in the cytoplasm. Under conditions of low oxygen, HIF-1α in control as well as in $CoCl_2$-treated cells was found in the nuclei. It has been reported that HSP90 played an important role to regulate the stability of HIF-1α by interacting with it [29]. Kubis et al.' investigation shown myocytes contained high HSP90 content and expression of HSP90 was independent of oxygen concentration. Above data indicate that the stability of HIF-1α is regulated not only by ubiquitin-proteasome pathway but also by HSP90 which interacts with HIF-1α and masks the NLS motif of HIF-1α during normoxia and hampers consequently nuclear import of it. However, hypoxia can induce HIF-1α accumulation in the cytoplasm of myoblasts and its translocation into nucleus.

What's the role does HIF-1α play in myogenesis? Ono et al. [30] analyzed the role of HIF-1α in myoblast differentiation by examining the expression of HIF-1α and by knocking down HIF-1α of C2C12 myoblasts with small interfering RNA (siRNA). Full differentiation into multinucleated myotubes was obtained by culturing 80—90% confluent C2C12 cells in DM. Myotube differentiation markers, MHC and myogenin were detectable on day 1 and day 3 after the cells were placed in DM. The level of HIF-1α protein also increased from day 1 to day 3 under the differentiating culture condition. HIF-1α accumulated in the nucleus of myogenin-positive myoblasts on day 1 of differentiation. At a more maturated stage on day 3, HIF-1α was expressed both in the nucleus and in the cytoplasm. However, the amount of HIF-1α mRNA was constant regardless of the culture conditions. They also demonstrated that HSP90 protected HIF-1α from

proteasome degradation during myogenic differentiation of C2C12 cells. HIF-1α knockdown led to a significant reduction in MHC expression. In addition, multinucleated myotubes were almost undetectable in the cells treated with HIF-1α siRNA. Taken together, these data indicated that HIF-1α supported myoblast differentiation and might be one of the crucial transcriptional factors in the regulation of C2C12 myogenesis.

Hypoxia affects myoblasts proliferation and differentiation through HIF-1 signaling pathway

Hypoxia signal can quickly activate $MAPK^{p38}$, and prolonging hypoxia $MAPK^{ERK1/2}$ can be activated, existing in phosphorylation form. Activated $MAPK^{ERK1/2}$ phosphorylate HIF-1α and lead to increase HIF-1α mediated transcriptional activity of target genes [31]. The HIF-1 signaling pathway has been regarded as a central signaling pathway involved in hypoxia-induced responses. HIF-1 can up-regulate downstream target genes and plays a pivotal role in the process of hypoxia signal transduction in both physiology and pathophysiology. Many of target genes activated by HIF-1 are involved in cell proliferation, survival, and differentiation, including erythropointin (EPO), vascular endothelial growth factor (VEGF), platelet-derived growth factor-β (PDGF-β), basic fibroblast growth factor (bFGF), and others [32]. Ogilvie et al. [33] reported that the EPOR was expressed on myoblasts and can mediate a biological response of those cells to treatment with EPO. They also observed that EPO could stimulate the proliferation of myoblasts and regulate the terminal differentiation of myoblasts, and alter the pattern of expression of the MyoD family of transcription factors during muscle differentiation. These data suggested that EPO might have a potential role in muscle development or repair. Under hypoxia conditions the expression of genes of VEGF, Ptx3, FGF8b and so on were increased, and these genes were involved in regulation of proliferation and differentiation of myoblasts.

Goda et al. [34] reported that HIF-1α was essential for cell cycle arrest during hypoxia. They shown that hypoxia caused a HIF-1α-dependent increase in the expression of the cyclin-dependent kinase inhibitors p21 and p27, and hypophosphorylation of Rb in hypoxia was also HIF-1α dependent. These data indicated that HIF-1α was a major regulator of cell cycle process during hypoxia. HIF-1α participated in regulating the expression or activities of cell cycle regulators which exist in G_1/S checkpoint, such as cyclins, CDKs, CDKIs, and influenced cell proliferation.

Is HIF-1 signaling pathway the only hypoxia-sensitive regulation mechanism?

Notch signaling pathway being involved

Recently, Gustafsson et al. [35] reported that hypoxia blocked differentiation of neuronal and myogenic progenitors and maintained the undifferentiated state, which required functional Notch signaling. Notch signaling often functions to maintain the stem/progenitor cell state, for example in myogenesis and hematopoiesis. Gustafsson et al.'s results shown that hypoxia led to recruitment of HIF-1α to a Notch-responsive promoter and elevated expression of Notch downstream genes, such as MyoD. Their observations suggested that the primary link between hypoxia and Notch signaling involved an interaction between Notch ICD and HIF-1α.

TGF-β/Smad signaling pathway being involved

Recently, there were some articles described that TGF-β/Smad signaling pathway emerged to participate in regulating the hypoxia-induced responses. Transforming growth factor-β (TGF-β) profoundly influenced the differentiation of many cell types of mesenchymal origin, including preadipocytes [36], osteoblasts [37], and myoblasts [38]. TGF-β inhibited the induction of muscle-specific gene expression and myotube formation without affecting cell proliferation in cultured myoblasts [39,40]. The current model for the TGF-β signal transduction pathway maintains that TGF-β acts through a heteromeric complex of serine/threonine kinase receptors. Ligand binding to the receptors complex results in C-terminal phosphorylation of Smad2 and Smad3. Each of the two receptor-activated Smads forms heteromeric complexes with Smad4. The Smad complexes then translocate into the nucleus and activate gene expression. Smad3 is an important signaling transduction factor in TGF-β signaling pathway. Liu et al. described that TGF-β inhibited myogenic differentiation through Smad3-mediated transcriptional repression [41,42]. Zhu et al.'s results indicated that TGF-β inhibited myogenesis, at least in part, via Smad pathway, and the expression of myogenin and MEF2D decreased in C2C12 treated with TGF-β

[43]. In Akman et al.'s report, it was demonstrated that exposure of endothelial cells to 1% O_2 increased messenger RNA and protein levels of TGF-β2 as well as messenger RNA levels of the TGF-β2 typeII membrane receptor and induced HUVEC to autocrine TGF-β2 production [44], and caused phosphorylation of Smad2/Smad3 which translocated subsequently into nuclear and initiated downstream signaling [45]. It has been demonstrated that both TGF-β/Smad and hypoxia signaling pathways could synergize in the regulation of VEGF gene and EPO gene expression at the transcriptional level [46—48]. Zhu et al. evaluated the mechanisms of inhibition of adipocyte differentiation by hypoxia and TGF-β in human and murine marrow stromal cells and the role of TGF-β/Smad signaling in the inhibition of adipocytogenesis by hypoxia. Hypoxia activated the TGF-β/Smad signaling pathway, and the TGF-β intracellular signaling molecule Smad3 was necessary for the inhibition of adipocytogenesis by both TGF-β and hypoxia. Their findings indicated that TGF-β/Smad signaling was required for the inhibition of adipocyte differentiation by hypoxia [49]. These data suggest that Smad may play an important role in hypoxia-induced responses, and however, the detailed molecular mechanism on this process as well as cross-talking between two pathways each other need to be further investigated.

Maybe exist some HIF-1-independent signaling pathways

In Yun et al.'s [50] experiments, they found that no significant changes in myogenic differentiation at 21% O_2 when they infected proliferating C2C12 cells with retrovirus containing an O_2-insensitive and constitutively active form of HIF-1α. Their observations indicated that the HIF-1 pathway did not play an important role in the hypoxic inhibition of myogenesis and the hypoxic inhibition of myogenesis was independent of HIF-1α. However, this condition need to be further proved because Yun's experiments were conducted under normoxic conditions under which the mechanism of HIF-1α stability in myoblasts depended on interaction with HSP90 and its combination with HSP90 hampered nuclear import of HIF-1α. Hypoxia decreased the binding of HIF-1α to HSP90 and promoted nuclear translocation of HIF-1α. Hypoxia could cause the activation of NF-κB and the phosphorylation of its inhibitory subunit, IκB-α. Activated NF-κB translocated into the nucleus, bound specific κB moieties, and initiated gene transcription [51]. The HIF-1-independent pathways might include various post-transcription responses, post-translation modification and changes in subcellular proteins transportation, and so on. Some additive responses to hypoxia caused by modification in expression of some growth factors by hypoxia were not controlled by the classical hypoxic pathway, which indicated that other HIF-1-independent pathways could be involved in these regulation.

In addition, using the advanced technology of macroarray such as gene chip, protein chip to highly throughput screen differential expression genes in myoblasts under hypoxia conditions compared with nomorxia conditions, we will explore and analyze signaling pathways involved in adaptive responses to hypoxia so as to investigate the effects of hypoxia on proliferation and differentiation of myoblasts.

Conclusion

Adaptive response to hypoxia bear specific based on cytotypes. For many stem cells moderate low oxygen concentration can promote proliferation of stem cells, and influence differentiation potential and determination of stem cells [52—57]. Up to now, the studies about effects of hypoxia on proliferation and differentiation of myoblasts are very few. Studying effects of hypoxia on myogenesis may provide a good model which is used to explore skeletal muscle cells' behavior under physiological hypoxia during embryo development or extreme hypoxia conditions attributed to exercise as well as pathological damage. According to reports published we presume that physiological hypoxia promoting stem cells proliferation and differentiation should be an evolution mechanism benefiting for tissues or organs shaping and functions established during development. And this is also the physiological foundation for self-renewal. Under the adverse conditions caused by diseases, damage and quick metabolization *in vivo*, cell cycle arrest and differentiation inhibition of stem cells may be a protective stress response, which could prevent stem cells from differentiating into functional cells to be damaged. This process decrease minimally organisms' damage. It has been shown that the response to hypoxia was adaptive. This adaptive mechanism might benefit for maintaining ''stem cells pool'' *in vivo* when individual exposed in physiological hypoxia for avoiding excess terminal differentiation of stem cells so as to retain potential of self-renewal, and on the other hand, stem cells could perform renewal to repair tissues and to reestablish functions after a

period of pathological changes. It is summarized that hypoxia-induced response is a kind of stress response.

To date, the mechanisms and signaling pathways involved in effects of hypoxia on survival, proliferation and differentiation of stem cells are still unclear. And some problems exist, for example, the standardization of choosing oxygen levels in which myoblasts are cultured; the limit of definition of hypoxia, that is, differentiation between physiological hypoxia and pathological hypoxia; whether the results derived from experiments *in vitro* are consistent with that in microenvironment *in vivo*; synergistic cooperation between hypoxia and other inner factors *in vivo*. It is take on huge advantage and perspective for myoblasts to be involved in muscle damage and repair as well as treating many degenerative diseases. Accordingly, it is very significant for theory and clinic to investigate the effects of hypoxia on proliferation and differentiation of myoblasts. Meanwhile, deep studying and further revealing the signaling pathways involved in mechanism will provide evidences or references for looking for novel targets for stem cells therapy and drug treatment.

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