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COMMENTARY

# Regulation of myogenesis by environmental hypoxia

**Michèle Beaudry[1,*], Magdalena Hidalgo[1,2], Thierry Launay[3,4], Valérie Bello[2] and Thierry Darribère[2,*]**

## ABSTRACT

In aerobic organisms, oxygen is a critical factor for tissue and organ morphogenesis from embryonic development throughout the adult life. It regulates various intracellular pathways involved in cellular metabolism, proliferation, cell survival and fate. Organisms or tissues rapidly respond to changes in oxygen availability by activating complex signalling networks, which culminate in the control of mRNA translation and/or gene expression. This Commentary presents the effects of hypoxia during embryonic development, myoblasts and satellite cell proliferation and differentiation in vertebrates. We also outline the relationship between Notch, Wnt and growth factor signalling pathways, as well as the post-transcriptional regulation of myogenesis under conditions of hypoxia.

**KEY WORDS: Hypoxia, Myogenesis, Signalling**

## Introduction

Since the experiments of Priestley (1774) it has been established that most organisms, including vertebrates, invertebrates, yeasts and bacteria, require molecular oxygen ($O_2$) for their survival. Oxygen is needed for biochemical reactions and is used by mitochondria to produce ATP. Oxygen is also an environmental and developmental signal involved in processes, such as energy homeostasis, development and progenitor cell differentiation.

Eukaryotic organisms regulate homeostasis of molecular oxygen in adult muscles by multiple means, such as through metabolic responses, contractile function, excitation–contraction coupling and switching of muscle fibre types. The mitochondria, as a site of oxygen consumption and reactive oxygen species (ROS) production, play a central role in muscle oxygen sensing and homeostasis (for reviews see, for example, Favier et al., 2015; Movafagh et al., 2015). However, these aspects will not be addressed in this Commentary. Instead, we discuss here the effects of oxygen availability in the regulation of myogenesis, the biological process that leads to the formation of muscle tissue, which occurs during embryonic, fetal and postnatal development, as well as during muscle repair and regeneration in adults. After introducing hypoxia and the hypoxia-inducible transcription factors, we present the effects of hypoxia during embryonic development, myoblast and satellite cell proliferation and differentiation. We also outline here the relationship between Notch, Wnt and growth factor signalling pathways, as well as the post-transcriptional regulation of myogenesis under hypoxia.

## Hypoxia

The partial pressure of oxygen ($P_{O2}$) is ∼21.2 kPa in atmospheric air at sea level, corresponding to the inspired fraction of 20.93% $O_2$. However, in tissues, the local oxygen pressure is much lower. For example, in adults, oxygen pressure in arterial blood is 14 to15 kPa (12% $O_2$) and its partial pressure in tissues is 3 to 5 kPa (3% $O_2$) with important local variations. The physiological $P_{O2}$ levels in skeletal muscles are 4.6–5.33 kPa, that is ∼5% $O_2$, and even lower in working muscles (for a review on partial oxygen pressures of human tissues, see Carreau et al., 2011). Mostly, the different thresholds of oxygen availability are 2 to 9%, 1 to 5%, ≤1%, ≤0.1% for physiological hypoxia, mild hypoxia, hypoxia and anoxia, respectively. They correspond to a $P_{O2}$ of 15–68 mmHg, 8–38 mmHg, ≤8 mmHg and ≤0.08 mmHg, respectively (Koh and Powis, 2012).

Hypoxia is defined as a lower $P_{O2}$ (≤1% $O_2$) and results in a reduced oxygen availability, both at the tissue and cellular levels. Reduced oxygen availability can occur either during acute hypoxia or during chronic hypoxia. Acute hypoxia refers to a sudden or rapid depletion in available oxygen, ranging from fractions of a second to minutes. At the cellular level, acute hypoxia induces a fast response that involves the post-translational modifications of proteins that are already present. By contrast, chronic hypoxia is characterized by the prolonged exposure to reduced oxygen availability, ranging from hours to days. At the cellular level, chronic hypoxia induces long-lasting responses, including gene activation and mRNA and protein expression; these are initiated by hypoxia-specific factors as discussed below.

## Factors induced by hypoxia

It is known that, under hypoxia, kidneys secrete the glycoprotein hormone erythropoietin to increase the production of red blood cells (for a review, see Jelkmann, 2011). Studies of *Epo* gene regulation have resulted in the identification of a cis-acting DNA sequence in the 3′-flanking region of the gene, named the hypoxia response element (HRE) (Beck et al., 1991). Subsequent work using oligonucleotide affinity chromatography based on the *Epo* HRE identified a complex bound to this DNA element (Semenza et al., 1991). This complex was named hypoxia-inducing factor (HIF) and has been shown to comprise a dimeric transcription factor composed of an oxygen-dependent subunit, that is HIF-1α or HIF-2α, and the oxygen-independent subunit HIF-1β (see Box 1).

HIF-1α exhibits a stability and activity that is regulated by oxygen availability. During normoxia, HIF-1α is continuously degraded and has a half-life of less than five minutes (Jaakkola et al., 2001). The family of oxygen-dependent prolyl hydroxylases (PHDs) catalyse hydroxylation of two key proline residues (P402 and P564) located in the oxygen-dependent degradation domain (ODDD) of HIF-1α (Figs 1 and 2; Jaakkola et al., 2001). Mammalian genomes encode three closely related PHDs (PHD1, PHD2 and PHD3; also known as EGLN2, EGLN1 and EGLN3, respectively). PHDs require 2-oxoglutarate, $Fe^{2+}$ and molecular oxygen to function (Fig. 2). PHD1 is detectable in muscle cells, both at the mRNA and protein levels, and loss of PHD1 lowers oxygen consumption in muscle (Aragonés et al., 2008). The hydroxylation of proline residues in HIF-1α by PHDs marks the

[1]Université Paris13 Sorbonne Paris Cité, EA2363, UFR-SMBH, Laboratoire Hypoxie et poumons, Bobigny 93017, Cedex, France. [2]UPMC Sorbonne Universités (Université Pierre et Marie Curie), UMR CNRS 7622, Laboratoire de Biologie du Développement, Paris 75252, Cedex 05, France. [3]Université Paris-Descartes Sorbonne Paris cité, 75015 Paris, France. [4]Université d'Evry, Unité de Biologie Intégrative Appliquée à l'Exercice EA 7372, 9100 Evry, France.

*Authors for correspondence (mbeaudry@yahoo.fr; thierry.darribere@upmc.fr)

T.D., 0000-0008-1890-625X

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**Box 1. The HIF proteins**

The HIFs proteins belong to the Per-ARNT-Sim (PAS) subfamily of basic helix-loop-helix (bHLH) family of transcription factors. The mammalian genomes encode three HIF-α isoforms, each manifesting oxygen-sensitive activity: HIF-1α, HIF-2α and HIF-3α. Their bHLH domain allows DNA binding, whereas the PAS domain mediates protein–protein interaction. Both domains are also involved in dimerization of the α and β subunits. HIF-1α is the most ubiquitously expressed and best characterized protein of the family. HIF-2α shares 48% amino acid sequence identity with HIF-1α and has a similar protein structure (Fig. 1). HIF-2α is only expressed in few cell types, mainly endothelial and lung epithelial cells, and data regarding its role in myogenesis is relatively scarce. HIF-2α can dimerize with HIF-1β, and the resulting transcription factor HIF-2 can activate expression of its target genes. HIF-1 and HIF-2 are non-redundant and have distinct target genes and mechanisms of regulation. It appears that HIF-1 has key roles in the initial response to hypoxia, whereas HIF-2 drives the hypoxic responses during chronic hypoxic exposure, at least in tumour cells (Holmquist-Mengelbier et al., 2006; Koh et al., 2011). HIF-3α, originally called IPAS, is less characterized. The human *HIF3A* gene has 19 predicted variants that result from different promoters and transcription initiation sites, as well as alternative splicing. Among these variants, eight are expressed in different tissues at different times, and are differentially regulated by hypoxia and other factors (Duan, 2016). The existence of such a range of HIF-3α variants makes it extremely difficult to analyse their specific functions. However, it appears that full-length HIF-3α also activates a transcriptional programme in response to hypoxia. In addition, some HIF-3α variants act as dominant-negative regulators of HIF-1 and/or HIF-2, whereas other variants can inhibit their functions by competing for the common HIF-β subunit (for a review, see Duan, 2016).

The β-subunit, also known as the aryl hydrocarbon receptor nuclear translocator (ARNT), has three paralogues HIF-1β, HIF-2β and HIF-3β, which are all nuclear proteins. In contrast to HIF-1α, the expression of the β-subunit is constitutive, both at mRNA and protein levels, and is therefore not regulated by hypoxia.

HIF-1 functions as a master regulator of oxygen homeostasis induced by hypoxia in all nucleated cells, whereas HIF-2 has a more restricted distribution in adult cells.

protein for ubiquitylation and subsequent destruction by the von Hippel–Lindau (VHL) tumour suppressor (Ohh et al., 2000). In hypoxia, PHDs lose their activity, thereby preventing HIF-1α hydroxylation and allowing its rapid accumulation in the cytoplasm of the cell (Fig. 2). Thereafter, HIF-1α is translocated into the nucleus where it binds to the HIF-1β subunit to form the HIF-1 transcription factor (Box 1). HIF-1 then binds to the 5′-(R)CGTG-3′ consensus sequence (with R referring to A or G) of HREs (cis-acting DNA sequences located within the promoters, introns and 3′ enhancers of a large number of oxygen-regulated target genes). Following binding to HREs, HIF-1 recruits transcriptional co-activators such as members of the p300-CBP family and transcription is initiated by the RNA polymerase II machinery (Fig. 2). Chromatin immunoprecipitation coupled to high-throughput sequencing and mRNA microarray experiments have revealed that HIF-1 and HIF-2 bind to ∼500 high-affinity target sites across the genome of human breast cancer cells (MCF-7) (Schödel et al., 2011). The exact contribution of HIF-2 in muscle has to be demonstrated (Favier et al., 2015).

Although the role of HIF-1 is relatively well studied in the context of cancer biology, much less in known regarding its role during hypoxia in developmental biology as discussed in the next section.

## The effects of hypoxia during development

Normal mammalian development occurs in low-oxygen conditions with oxygen concentrations ranging from 1% to 5% in the uterine environment (Dunwoodie, 2009). If oxygen availability decreases, hypoxia causes growth retardation and affects placental and heart development, as well as chondrogenesis and bone formation (for a review, see Dunwoodie, 2009). However, currently, our understanding of the effects of hypoxia on myogenesis during early mammalian development is limited.

During development, segmentation of the vertebrate body is driven by the regular repeated formation of somites from the paraxial mesoderm. Somites are transient mesodermal structures that form as balls of epithelial cells and give rise to the vertebrae and ribs, as well as the dermis of skin and muscles in the body, apart from head muscles (for a review, see Musumeci et al., 2015). The medial portion of somites gives rise to deep back muscles, and the lateral somites form body wall and limb muscles. During embryogenesis, muscle cells develop in several steps: first, differentiation of somites into dermomyotome-containing myogenic precursors, then proliferation of myogenic precursors and their determination into myoblasts, followed by proliferation of myoblasts and their differentiation and fusion into myotubes, and, finally, maturation of myotubes into myofibres (Fig. 3). After birth, myofibres grow by fusion of additional myoblasts and an increase in the size of muscle fibres. The precursor cells that are committed to myogenic fates are under the control of the paired-box transcriptional factors Pax3 and Pax7 (Relaix et al., 2005). Cells express Pax3 in the early embryo and Pax7 in the foetus and adult. Both are essential for embryonic and adult myogenesis, and specifically for the downstream activation of myogenic regulatory factors (MRFs) such as MyoD (also known as MyoD1), Myf5, myogenin and MRF4 (also known as MYF6). MRFs control gene expression to specify muscle progenitor cells for the muscle fate and to promote myogenic differentiation (Fig. 3; for a review, see Bentzinger et al., 2012). In addition, microRNAs (miRNAs), a class of post-transcriptional regulators, are also essential for myogenesis (Fig. 3).

During embryonic and adult myogenesis, muscle progenitors reside in low-oxygen environments before local blood vessels and differentiated muscle form (Dunwoodie, 2009). Mice knockouts of HIF-1α and HIF-β are embryonically lethal with prominent defects in brachial arches and cranium, and a reduction in somite number (Iyer et al., 1998; Ryan et al., 1998). Furthermore, their somites are morphologically abnormal and misaligned with respect to the midline of the body (Kotch et al., 1999). Mice in which HIF-1α has been deleted fail to proceed through myofibril formation during embryonic stages, as they succumb to vascular and placental defects before muscle development is complete (Majmundar et al., 2015). HIF-2α-deficient mice exhibit retinopathy, hepatic steatosis, cardiac hypertrophy, azoospermia and mitochondrial abnormalities, as well as muscular defects. In muscles, myofibrils appear to be distorted or compressed, with lipids localized within cells, somewhat similar to in dystrophic muscles (Scortegagna et al., 2003). These data suggest that in normal mammalian development, HIF might be required for the formation of somites from which myogenic progenitor cells are derived. To our knowledge, there is no additional information regarding the function of HIF, especially during hypoxia; however, some data exist for other vertebrates.

In zebrafish, hypoxia exposure significantly reduces body size, as is the case in mammalian foetuses. Hypoxia also causes delays in embryonic development, as suggested by a reduced somite number and delayed myogenesis without any patterning abnormalities (Kajimura et al., 2005). In amphibians, hypoxia does not affect somitogenesis; instead it results in a defect in the accumulation of muscle-specific proteins, such as the myosin heavy chains (MyHCs) and an endoplasmic reticulum (ER) membrane protein of unknown

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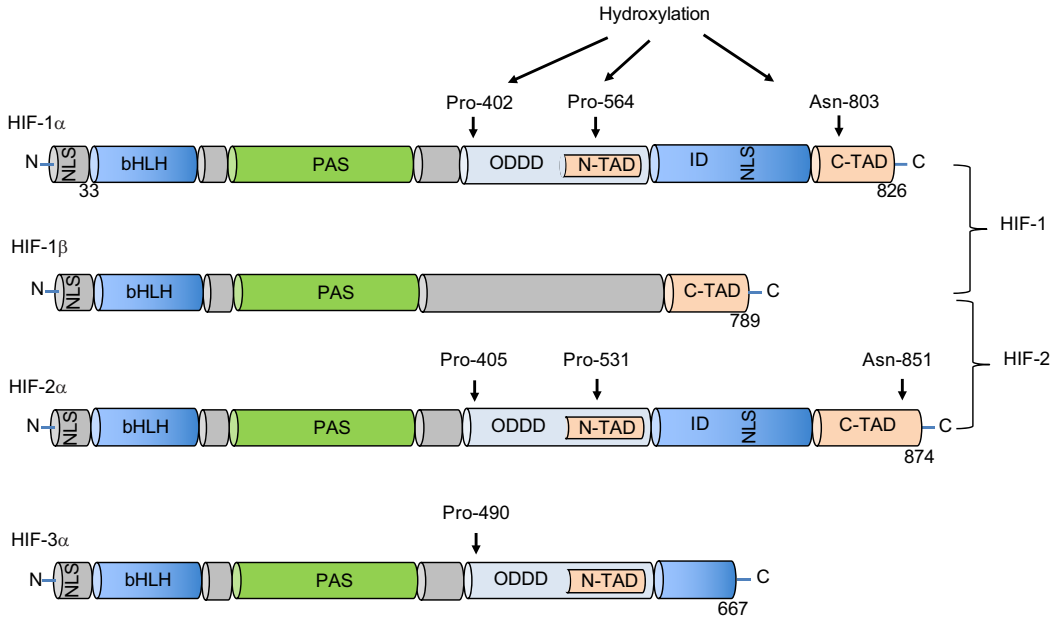


**Fig. 1. Domain structure of human HIF subunits.** Shown here are the schematic structures of HIF-1α (826 amino acids), HIF-2α (874 amino acids) and HIF-3α (667 amino acids), as well as HIF-1β (789 amino acids). The N-terminal half of HIFs consists of basic helix-loop-helix (bHLH) and Per-ARNT-Sim homology domains (PAS) that are required for heterodimerization and DNA binding, respectively. HIF-1α, HIF-2α and HIF-3α also possess an oxygen-dependent degradation domain (ODDD). The ODDD is required for oxygen-dependent hydroxylation and degradation. The C-terminal region of HIF-1α and HIF-2α contain two terminal-transactivation domains (TADs); these are designated N-TAD and C-TAD and are separated by an inhibitory domain (ID). N-TAD and C-TAD are responsible for the transcriptional activity of HIF-1α and of HIF-2α under hypoxia. The transcriptional activity of the C-TAD domain is associated with the binding of transcriptional co-activators, including the CREB-binding protein (CBP)-p300. The co-activators are then able to bind HIF-1 and subsequently the complex initiates transcription under hypoxia. The N-TAD domain is responsible for stabilizing HIF-1α against degradation. The NAD domain overlaps the ODDD, it links the transcriptional activity of HIF-1 with the stabilization of the protein. HIF-3α and HIF-1β contain only one TAD. HIF-1α, HIF-2α and HIF-3α are potentially hydroxylated at two proline residues (Pro), which induces binding of von Hippel–Lindau protein (pVHL) (see Fig. 2). Hydroxylation of an asparagine residue (Asn) located in the C-TAD of HIF-1α and HIF-2α inhibits their interaction with p300 and CBP. NLS, nuclear localization signal. Adapted with permission from Prabhakar and Semenza, 2012.

identity in somites without affecting the expression of MyoD and MRF4 (Hidalgo et al., 2012).

These data strongly suggest that the HIF transcription factor is important in embryos to enable cellular survival of muscle progenitors in somites that develop in a low-oxygen environment. To go further and specify the effect of hypoxia on embryonic myogenesis, it will be important to develop conditional inactivation approaches to disrupt HIF genes specifically in myoblasts and skeletal muscles. A transgenic mouse line that expresses Cre recombinase under the control of a skeletal-muscle-specific promoter could be a useful tool to identify the molecular mechanisms through which HIFs regulate myogenesis and embryonic muscle formation under hypoxia (Heidt and Black, 2005). Although currently there is only a small amount of data on the role of HIF in embryonic myogenesis under hypoxia, a number of studies have addressed its role in *in vitro* myogenesis as discussed below.

## Hypoxia-mediated regulation of myoblast proliferation and differentiation *in vitro*

Although studies addressing the effects of hypoxia on myogenesis *in vivo* are limited, experiments have been performed using the mouse C2C12 and rat L6 skeletal muscle cell lines, and primary human myoblasts. In these systems, conversion of myoblasts into myotubes represents a well-established and robust *in vitro* differentiation model to study myoblast proliferation and differentiation, as well as postnatal muscle formation.

Myogenesis of C2C12 and L6 cells, as well as of human myoblasts, is inhibited below 2% $O_2$, with maximal inhibition at 0.01% $O_2$ without any apparent effects on cell viability, whereas myogenesis is not affected at 5% oxygen (Yun et al., 2005; Launay et al., 2010). Myofibres that form at 2% $O_2$ appear less substantial, are poorly organized and have smaller cross-sections than their counterparts that develop at 21% $O_2$ (Yun et al., 2005). These results suggest that the degree of inhibition of myogenesis is proportional to the severity of hypoxia, and that 5% oxygen appears to be the optimal condition for muscle cell differentiation *in vitro* (Launay et al., 2010; Yun et al., 2005; Hidalgo et al., 2014). Below 1% $O_2$, C2C12 cells undergo a proliferation arrest and accumulate in the G1 phase of the cell cycle. These cells fail to upregulate both the cyclin-dependent kinase p21 (also known as CDKN1A) and the nuclear protein pRb (also known as RB1), which are both known to participate in cell cycle progression, thereby preventing both the permanent cell cycle withdrawal and terminal differentiation (Di Carlo et al., 2004). Furthermore, hypoxia strongly inhibits myoblast differentiation as it results in decreased expression of MyoD, Myf5, myogenin and MyHCs, which impedes formation of multinucleated myotubes (Di Carlo et al., 2004). Here, the decrease in MyoD occurs both at transcriptional and protein levels. At the protein level, hypoxia induces MyoD degradation; this is proportional to the severity of hypoxia and mediated by the ubiquitin-proteasome pathway (Di Carlo et al., 2004; Yun et al., 2005). Interestingly, partial myoblast differentiation still occurs after 1 to 3 days of exposure to 0.01% $O_2$. Under these conditions, *MyoD* is expressed, but the myofibres are smaller than those that have recovered after hypoxia at 2% or 0.5% $O_2$. Thus, myogenesis can occur in cells recovering from 2% or 0.5% $O_2$, but only to some extent when they have been exposed to extreme hypoxia (0.01% $O_2$) (Yun et al.,

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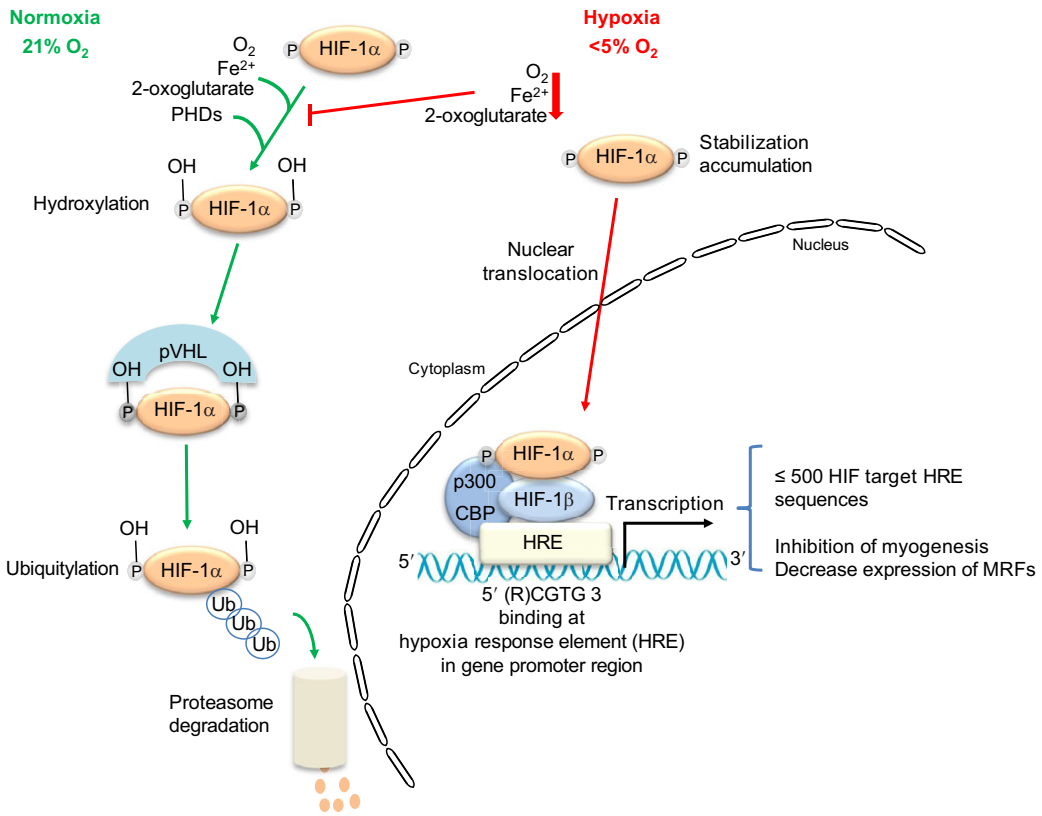


**Fig. 2. Regulation of HIF-1α by prolyl hydroxylation and proteasomal degradation.** Under conditions of normoxia and in the presence of molecular oxygen, 2-oxoglutarate and $Fe^{2+}$, HIF-1α is hydroxylated on residues P402 and P564 (indicated by P, see Box 1) by prolyl hydroxylases (PHDs). This results in the binding of pVHL, which then mediates ubiquitylation (Ub) of HIF-1α and its subsequent proteasomal degradation. Under hypoxic conditions, $O_2$, $Fe^{2+}$ and 2-oxoglutarate become limited, thereby attenuating HIF-1α hydroxylation. HIF-1α therefore remains stable, accumulates in the cytoplasm and is subsequently translocated into the nucleus, where it binds to the HIF-1β subunit, forming the HIF-1α–HIF-1β dimer (HIF-1). HIF-1 interacts with hypoxia response elements (HREs) within the promoters of target genes and recruits transcriptional co-activators such as p300-CBP for transcriptional activity. In normoxia, the pathway involving pVHL regulates HIF-1α stabilization, whereas hypoxia regulates HIF-1α transactivation. p300-CBP, p300 and CREB-binding proteins.

2005). It is noteworthy that myoblast differentiation is not irreversibly inhibited by hypoxia. Myoblasts regain their capacity to proliferate and differentiate when normal oxygen levels are restored. Thus, although myogenesis is strongly inhibited at 0.5% $O_2$, overexpression of MyoD, or of myogenin alone restores myogenesis to a similar level to that in normoxia (Yun et al., 2005). Furthermore, myoblasts exposed for 3 days at 0.5% $O_2$ are able to fuse and form myotubes, suggesting an adaptation of myogenesis in prolonged hypoxia (Yun et al., 2005).

Taken together, these finding demonstrate that hypoxia negatively regulates myogenic proliferation and differentiation by inhibiting the expression of MRFs and exit from the cell cycle. These effects of hypoxia on myoblasts share certain features with the effects on satellite cells, which are believed to be stem cells

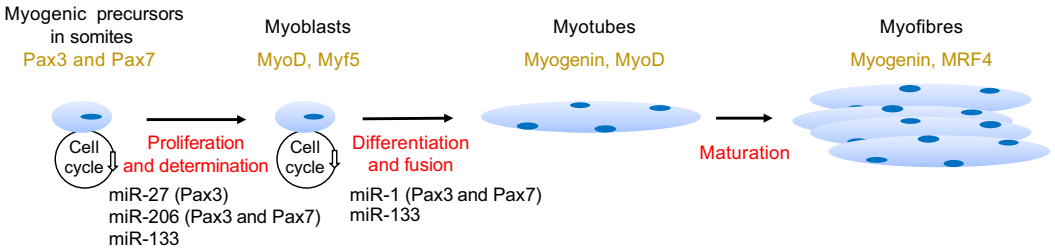


**Fig. 3. A simplified schematic representation of myogenesis.** The phases of myogenesis and main molecular factors involved in the development of the mature muscle fibre are shown. Muscles of the body, with the exception of some head muscles, are derived from cells of the somites. This population of progenitors gives rise to muscle cells and the satellite cells residing in postnatal muscles (for a review, see Relaix et al., 2005). Muscle progenitors in somites and myoblasts exit from the cell cycle, no longer proliferate and begin to differentiate. This is controlled by a hierarchy of transcription factors that regulates the progression of precursors in somites through the myogenic lineage. Pax3 and Pax7 are involved in myogenic determination, whereas Myf5 and MyoD commit myoblasts to the myogenic programme. The terminal differentiation requires both myogenin and MRF4 to trigger the transcription of genes encoding for specific muscle proteins, such as skeletal muscle actin, heavy and light chains of myosin, and muscle isoforms of tropomyosin and troponin (for a review, see Musumeci et al., 2015). Indicated here are also some muscle specific miRNAs that are upregulated during the cell cycle exit of myogenic precursors and their differentiation into myotubes. Their target mRNAs are indicated in brackets (see Sayed and Abdellatif, 2011 and Horak et al., 2016 for detailed reviews).

for postnatal myogenesis and muscle regeneration, as described below.

### Effect of hypoxia on myogenic satellite cells

In 1961, electron microscopy made it possible for the first time to observe mononucleated cells intimately associated with myofibres of frog muscle (Mauro, 1961). These were named satellite cells and their existence was later confirmed in other animals, including humans (Kuang and Rudnicki, 2008). Satellite cells are stem cells that derive from embryonic progenitors of the dermomyotome and form a reservoir of precursor cells residing in an hypoxic microenvironment between the basal lamina and the plasma membrane of the muscle fibre, both in embryos and adults (Gros et al., 2005). Satellite cells can form myoblasts that will go through a similar myogenesis process to that observed during development. Thereby, they are responsible for postnatal muscle growth, muscle maintenance and muscle repair after damage either by exercise or disease.

In mice, the number of satellite cells is at a maximum at birth and then declines to ∼1–5% of this level in adults (Bischoff, 1994). In humans, there is little information on the number of satellite cells in muscles and of changes in satellite cell content from birth to old age. Nevertheless, it is known that the number of satellite cells decreases with age, leading to reduced proliferative and fusion capacity in aged muscle, which likely contributes to decreased regeneration capabilities.

The different cell states during myogenesis can be distinguished based on their expression of Pax7 and MyoD. Quiescent and self-renewing satellite cells express Pax7 but not MyoD, whereas proliferative satellite cells express both Pax7 and MyoD. During differentiation, satellite cells no longer express Pax7 but still have MyoD (Zammit et al., 2004). In cultured adult muscle fibres, satellite cells exit the fibres, proliferate and differentiate on the parent fibres. Moreover, they can also migrate onto other fibres in the culture, or adhere to the culture plate (Zammit et al., 2004).

*In vitro* studies have demonstrated that cultured myogenic C2C12 cells and primary myoblasts obtained from mice hindlimb muscles are maintained in an undifferentiated state during hypoxia by inhibiting their differentiation through the Notch or phosphoinositide 3-kinase (PI3K)–Akt signalling pathways (see below and Gustafsson et al., 2005; Majmundar et al., 2012). Hypoxia has been shown to induce quiescence of satellite cells that derive from mouse primary myoblasts of hindlimb muscles by upregulating Pax7 and by downregulating MyoD and myogenin (Liu et al., 2012). In cultured cells at 1% $O_2$, the overall rate of proliferation is not affected. By contrast, the asymmetric divisions that generate the self-renewing and proliferating progeny are increased. Conversely the asymmetric divisions that generate a differentiating and a proliferating progeny are decreased (Liu et al., 2012). Analysis of satellite cells derived from 31-month-old rats cultured at 3% $O_2$ indicate an increase in the number of large colonies of satellite cells. This stems from activation of cyclin-dependent kinases (CDKs), phosphorylation of Akt and downregulation of a CDK inhibitor (p27; also known as CDKN1B) (Chakravarthy et al., 2001). These data suggest that lower oxygen levels stimulate the proliferation of old satellite cells by enhancing their self-renewal potential. Hypoxia also stimulates proliferation of bovine satellite cells and promotes their myogenic differentiation, which involves the upregulation of MyoD (Kook et al., 2008). These observations are important for potential therapeutic stem cell transplantations, because the proliferation and differentiation abilities of satellite cells of mature myofibres decline with increasing age.

Another factor involved in myogenesis is the basic helix-loop-helix transcriptional repressor Bhlhe40, which has a role in the regulation of circadian rhythm, adipogenesis, fatty acid oxidation, apoptosis and cell proliferation (for a review, see Kato et al., 2014). *In vitro*, Bhlhe40$^{-/-}$ primary myoblasts show increased proliferation and impaired differentiation, whereas knockdown of Bhlhe40 delays regeneration of mouse muscle that has been subjected to freeze injury. Furthermore, Bhlhe40$^{-/-}$ mice exhibit increased cellular proliferation and a regeneration defect that is characterized by degenerated myotubes (Sun et al., 2007). These findings suggest that Bhlhe40 is required for postnatal myogenesis by promoting myogenic differentiation. Surprisingly, however, overexpression of Bhlhe40 mimics the effect of hypoxia as it has been reported to inhibit myoblast differentiation *in vitro* owing to its interaction with MyoD (Azmi et al., 2004).

Bhlhe40 is strongly induced by hypoxia (1% $O_2$) in satellite-cell-derived primary myoblasts where it mediates repression of myogenin expression through decreasing the transcriptional activity of MyoD. This is followed by the inhibition of myogenic differentiation (Wang et al., 2015). Conversely, inhibition of Bhlhe40 upregulates myogenin expression and promotes cell differentiation. Moreover, knockdown of Bhlhe40 rescues satellite cell differentiation under hypoxia (Wang et al., 2015). Finally, it is interesting to note that hypoxia induces Bhlhe40 expression independently of HIF-1α, and through a signalling pathway that involves the tetrameric transcription factor p53, which has a crucial role in cell cycle arrest and apoptosis (Wang et al., 2015).

Myoblasts are already being used in cell transplantation therapies to expand the existing satellite cell pool and to regenerate muscles, both in mouse models and human patients. However, only few of the transplanted myoblasts survive and contribute to new satellite cells, resulting in limited therapeutic applications (Kuang and Rudnicki, 2008). In contrast, hypoxia-conditioned myoblasts show improved homing and regenerative efficiencies when they are grafted into *mdx* mice, an animal model of Duchenne muscular dystrophy (DMD) that exhibits muscle degeneration (Liu et al., 2012). These myoblasts show reduced expression levels of MyoD, which decreases their ability to differentiate and so enhances their self-renewal potential after transplantation. Furthermore, the role of Bhlhe40 as a hypoxia-responsive factor suggests that inhibition of Bhlhe40 or p53 might facilitate muscle regeneration after injury and cell differentiation following transplantation.

### The complex relationship between Notch, Wnt and hypoxia

Besides the involvement of the above transcription factors in regulating myogenesis under hypoxia, factors that originate from outside of the cell, such as Notch and Wnt, are also mobilized in hypoxia. The *Notch* gene family encodes transmembrane receptors that in vertebrates directly interacts with their ligands Jagged and Delta, which are located on adjacent cells. The resulting activation of Notch induces its proteolytic cleavages and results in the release of the Notch intracellular domain (NICD), which enters the nucleus and forms a transcriptional complex with co-factors in order to activate Notch target genes (Fig. 4) to regulate cell fate determination during development and maintenance of adult tissue homeostasis.

Notch signalling is known to promote the transition of activated satellite cells to highly proliferative myogenic precursors and myoblasts. It also prevents myoblast differentiation into myotubes after injury to maintain the stem cell state (for a review, see Tsivitse, 2010). However, it has been shown that Notch is not overexpressed during hypoxia and is in fact inhibited in severe hypoxia (<0.5%) in C2C12 myoblasts (Yun et al., 2005). By contrast, another study has

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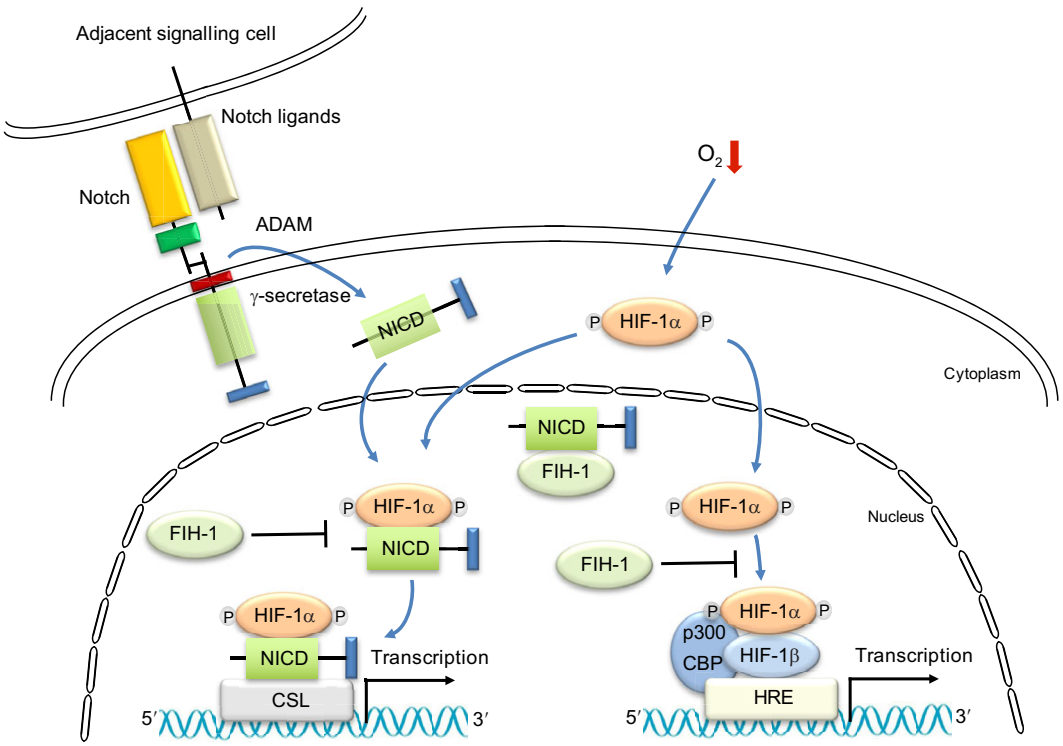


**Fig. 4. Interdependence between Notch and hypoxia signalling pathways.** The transmembrane Notch receptor interacts directly with Notch ligands located on adjacent cells. Activation of Notch results in two cleavage events catalysed by extracellular metalloproteases (ADAM) and intracellular γ-secretase, which release the Notch intracellular domain (NICD) in the cytoplasm. In the absence of NICD, the CSL transcription factor (also known as RBPJ, CBF-1, Suppressor of Hairless or Lag-1) represses transcription of Notch target genes. Following activation by NICD, CSL is converted into a transcriptional activator to stimulate transcription of the same genes. Upon hypoxia and activation of Notch, HIF-1α induces the hypoxia response pathway and potentiates Notch signalling through interactions with the NICD and the CSL transcription factor. In the presence of factor-inhibiting HIF-1α (FIH-1), HIF-1α becomes hydroxylated at asparagine (see Box 1). FIH-1 blocks the interaction of the C-TAD of HIF-1**α** with the transcriptional co-activator p300-CBP. NICD might sequester FIH-1, thereby preventing hydroxylation of HIF-1**α** and consequently enhancing recruitment of HIF-1α to HRE sites.

reported that hypoxia leads to inhibition of differentiation in satellite cells and C2C12 myoblasts (Gustafsson et al., 2005). Those authors also demonstrate that blocking of Notch signalling with a γ-secretase inhibitor counteracts this inhibition and restores differentiation under hypoxia. In addition, immunoprecipitation studies show that HIF-1α and NICD can physically interact (Gustafsson et al., 2005). Furthermore, chromatin immunoprecipitation analyses confirm the recruitment of HIF-1α to promoters of Notch-responsive genes in cells exposed to hypoxia, providing further evidence that hypoxia upregulates the expression of known Notch-target genes (Zheng et al., 2008). These data reveal that there are two modes of action of HIF-1α, at least *in vitro*; first, a canonical response whereby it dimerizes with HIF-1β to activate the transcription of hypoxia-responsive genes, and, second, through Notch signalling. In the latter mode of action, it dimerizes with NICD to activate Notch target genes (Fig. 4). In any case, HIF-1α physically interacts with the NICD and is recruited to Notch-responsive promoters; it therefore cooperates with Notch to maintain an undifferentiated phenotype in myogenic satellite and C2C12 cells under hypoxic conditions (Gustafsson et al., 2005). As Notch signalling is a major pathway involved in embryonic development, this interdependence between HIF and Notch presents the exciting possibility that Notch signalling might be positively modulated in hypoxic regions during myogenesis in the embryo and muscle repair in adults. It would certainly be of great interest to determine the implication of this interdependence *in vivo*.

Interestingly, an HIF-1-inhibiting factor (FIH-1; also known as HIF1AN) has been identified as a modulator of HIF-1α protein stabilization and of its transcriptional activity in response to changes in cellular oxygen concentration (Mahon et al., 2001). FIH-1 is an α-ketoglutarate-dependent dioxygenase that hydroxylates the asparagine residue (N803) in the C-terminal transactivation domain of HIF-1α (Fig. 1). FIH-1-mediated hydroxylation is reduced when oxygen is depleted, thereby allowing HIF-1α to interact with the transcriptional co-activator CBP-p300 (Mahon et al., 2001). FIH-1 also hydroxylates NICD at two asparagine residues that have been shown to be crucial for its transactivator function in C2C12 myoblasts and *in vivo* (Zheng et al., 2008). Consequently, FIH-1 negatively regulates Notch activity and accelerates C2C12 myoblast differentiation. The role of FIH-1 in the regulation of the Notch pathway suggests that there is a dynamic and complex interdependence between Notch and hypoxia. On the one hand, HIF-1α is recruited to NICD and so enhances its transcriptional activity. In this case, HIF-1α and NICD have additive effects on transcription. On the other hand, because FIH-1 has a significantly higher affinity for NICD than for HIF-1α, NICD might sequester FIH-1, thereby preventing hydroxylation of HIF-1α and consequently enhancing its recruitment to HRE sites (Fig. 4; Zheng et al., 2008; Wilkins et al., 2009). In both cases, Notch signalling enhances the adaptive response to hypoxia during myogenesis.

Embryonic myogenesis is orchestrated by signalling networks that originate from the ectoderm, the neural tube, the notochord and the intermediate mesoderm, which surround the somites (for a review, see Musumeci et al., 2015). In particular, Wnt proteins secreted by the dorsal regions of the neural tube have been

demonstrated to activate the expression of MyoD and Myf5, leading to myogenic lineage progression and differentiation (von Maltzahn et al., 2012). These factors are also involved in regulation of satellite cell differentiation, as well as in self-renewal of satellite cells (Brack et al., 2008). By exploring the role of HIF-1α during murine muscle development and regeneration, it has been shown that at low oxygen levels, HIF-1α inhibits myogenesis through repression of Wnt signalling. Conversely, as oxygen levels rise, the protein levels of HIF-1α diminish, resulting in increased Wnt activity and enhanced myogenesis (Majmundar et al., 2015).

Therefore, the complex relationship between Notch, Wnt and hypoxia is likely to regulate muscle stem cell behaviour in a mutually dependent manner. In hypoxia, HIF stimulation inhibits myogenesis through enhanced Notch activity. In normoxia, HIF degradation accelerates myogenic differentiation by derepressing Wnt. In addition to such an interplay between Notch, Wnt and hypoxia, growth factors, cytokines, hormones and other signalling molecules (hereafter referred to as growth factors) also control the response of muscle cells to hypoxia, as discussed below.

## Effect of growth factor signalling pathways on myogenesis during hypoxia

Several studies in muscles have shown that the PI3K–Akt–mTOR signalling axis has a role in muscle development, regeneration and hypertrophy; this is mediated through several pathways involved in protein synthesis, mitogenic activity, atrophy inhibition and the prevention of cell death (Wilson and Rotwein, 2007).

Activation of phosphatidyl inositol-4,5-bisphosphate-3-kinase (PI3K) regulates protein synthesis through the activation of a kinase cascade that includes the serine/threonine-specific Akt kinases and the mammalian target of rapamycin (mTOR). Akt was described for the first time in 1991 and then was named RAC (for 'related to A and C kinases') or protein kinase B (PKB) because it has a high homology with PKA and PKC (Bellacosa et al., 1991). Thus far, three isoforms, encoded by different genes, have been identified in mammals: Akt1, Akt2 and Akt3 (also known as PKBα, PKBβ and PKBγ, respectively). Although the three isoforms are expressed ubiquitously in mammals, their expression level varies depending on the cell type. Akt1 is expressed in all tissues, whereas Akt2 is enriched in insulin-sensitive tissues, such as muscle, adipose tissue and liver (Wu et al., 2011). Akt3 is preferentially expressed in brain, pancreas, lung and kidney. Akt proteins regulate protein synthesis and muscle growth through activation of the PI3K–Akt–mTOR pathway (Lai et al., 2004).

Although growth factors induce differentiation in normoxia, they stimulate myoblast proliferation in hypoxia through activation of the same receptors. Indeed, during exposure of rat L6 myotubes to hypoxia, activity of the PI3K–Akt–mTOR pathway decreases, and an elevation of protein degradation occurs, thus altering muscle protein homeostasis (Caron et al., 2009; Hidalgo et al., 2014). In mouse myoblasts, the inhibition of the PI3K–Akt–mTOR axis by hypoxia promotes cell proliferation and reduces myogenic differentiation (Ren et al., 2010). In fact, forkhead box O transcription factors (of which the isoforms FoxO1, FoxO3 and FoxO4 are expressed in skeletal muscle) are involved in muscle differentiation, and FoxO1 and FoxO3 can be induced by hypoxia (Kitamura et al., 2007). Upon their phosphorylation by Akt, FoxOs exit the nucleus and are inactivated in the cytoplasm. When they are dephosphorylated, FoxOs are transcriptionally active in nucleus, and, in the cytoplasm, bind to proteasome enzymes, thereby inducing protein degradation and inhibiting differentiation (Mammucari et al., 2007). Interestingly, it has been shown that FoxO1 interacts with the Notch pathway to activate Notch target genes (Kitamura et al., 2007). This interaction integrates hypoxia cues signalling through Notch with metabolic cues signalling through FoxO1 to regulate muscle differentiation. Another pathway involved in the cellular response to growth is the p38 mitogen-activated protein kinase (MAPK) pathway. In normoxia, the p38 MAPK family has a crucial role in myogenesis by activating transcription factors, such as MyoD and the myocyte enhancer factor-2 (MEF2), a transcription factor involved in terminal differentiation of myoblasts. Hypoxia induces a decrease of p38-MAPK-mediated phosphorylation in differentiating myoblasts, thus contributing to switching the effects of growth factors from inducing differentiation to proliferation (Ren et al., 2010). Moreover, it has been shown that hypoxia reduces Akt activation, that is, its phosphorylation by phosphoinositide dependent kinase 1 (PDPK1) at T308 and by the mammalian target of rapamycin complex 2 (mTORC2) at S473, in differentiating myoblasts by a mechanism that is controlled by HIF-1. Interestingly, the restoration of Akt activity in C2C12 cells under hypoxia with the expression of a constitutively active form of Akt (myrAkt) leads to the reestablishment of myogenic differentiation as it restores mTOR activity. In the same way, inhibition of mTOR by rapamycin treatment abolishes the effect of growth factors on myogenic differentiation. A decrease in the activity of the PI3K–Akt–mTOR pathway, which has been implicated in protein translation through ribosome subunit synthesis, is thus responsible for the loss of myogenesis-activating potential of growth factors under hypoxia, but not mitogenic activities (Ren et al., 2010). Under hypoxia, growth factors also activate the Ras–Raf–MEK–ERK signalling cascade, which results in an increased rate of the translation of *Hif1a* mRNA and phosphorylation of p300-CBP family proteins, leading to increased HIF-1α–p300 complex formation. Thus, this cascade enhances the transcriptional activity of the complex. In addition to these signalling pathways, post-transcriptional and translational proteins levels are also regulated by hypoxia as discussed below.

## Post-transcriptional and translational regulation under hypoxia

The understanding of muscle myogenesis was broadened with the discovery of microRNAs (miRNAs). miRNAs were first discovered in *Caenorhabditis elegans* (Lee et al., 1993) and are a class of evolutionarily conserved, non-coding RNAs of 20 to 24 nucleotides. They regulate gene expression post-transcriptionally by mostly targeting the 3′ untranslated regions (3′UTR) of complementary mRNA, and, less often, their 5′UTR regions, to induce their translational repression (Lee et al., 2009). In myoblasts, miRNAs (myomiRs) are involved in cellular regulation of proliferation, differentiation, apoptosis and regeneration (Goljanek-Whysall et al., 2012; Yu and Zuo, 2013). There are currently eight known myomiRs: miR-1, miR-133a, miR-133b, miR-206, miR-208a, miR-208b, miR-486 and miR-499 (Horak et al., 2016). The expression of myomiRs is controlled by MRFs; for example, miR206 is upregulated by MyoD and targets *Pax3* and *Pax7* mRNA (Fig. 3). Through this negative-feedback mechanism, MyoD facilitates progression towards differentiation. Other myomiRs that control myogenesis are involved in many aspects of skeletal muscle development (see Horak et al., 2016 for a detailed review).

*In vivo* injection of miR-1 or miR-133 at the one-cell stage of *Xenopus* embryos leads to highly disorganized somites that fail to develop segmented structures, suggesting that correct temporal expression and amounts of both miR-1 and miR-133 are required for

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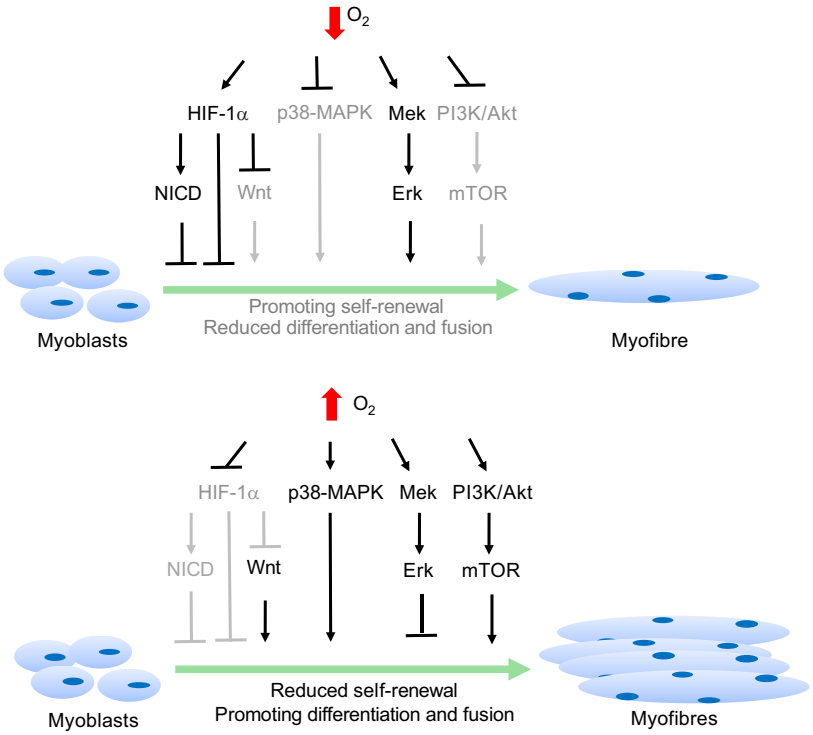


**Fig. 5. A model of the signalling pathways involved in response of muscle cell to environmental hypoxia.** When the availability of oxygen decreases ($\downarrow O_2$), HIF-1α accumulates in the nucleus. The cellular response to growth factors is altered, leading to the inactivation of the PI3K–Akt–mTOR and p38 MAPK pathways. In addition, Notch activity is enhanced, whereas Wnt signalling is repressed. Therefore, low oxygen levels contribute to promoting myoblast proliferation at the expense of their differentiation and fusion. When the availability of oxygen rises ($\uparrow O_2$), HIF-1α becomes degraded; this results in a decrease of Notch activity, increase of Wnt signalling and activation of the PI3K–Akt–mTOR and p38 MAPK pathways. The MEK–ERK pathway is also activated. Together, this results in limited cell proliferation in favour of differentiation and fusion through the upregulation of myogenic genes. Grey arrows highlight processes that are inhibited, whereas black highlight pathways that are induced.

proper somitogenesis (Chen et al., 2006). In mouse primary myoblasts isolated from hindlimb muscle, hypoxia maintains myoblasts in their undifferentiated state with a rapid increase in Pax7 expression, suggesting that post-transcriptional regulation of Pax7 might take place. As miR-1 and miR-206 can recognize the 3′UTR of *Pax7* mRNA and downregulate Pax7 protein production during normoxia (Chen et al., 2010), Liu et al. addressed their role and showed that miR-1 and miR-206 are downregulated in hypoxic cultures (Liu et al., 2012). These data also show that hypoxia upregulates protein levels of Pax7 through the downregulation of miR-1 and miR-206, as knockdown of miR-1 and miR206 abolishes the effect of hypoxia on Pax7. Interestingly, their findings also demonstrate that hypoxia activates Notch signalling, which leads to suppression of miR-1 and miR206 expression and to upregulation of Pax 7 (Liu et al., 2012).

Independently of any post-transcriptional regulation, gene expression can also be regulated at a translational level. Surprisingly, in vertebrates there are only few studies addressing the effect of hypoxia on mRNA translation in muscle cells. Recent data indicate that placing *Xenopus* embryos in hypoxic conditions reversibly decreases Akt phosphorylation. Analysis of muscle formation has provided strong evidence that hypoxia affects cell proliferation and muscle-specific protein accumulation in somites. These effects were independent of any variations in mRNA levels and did not involve expression of MyoD and MRF4 (Hidalgo et al., 2012).

## Conclusions

As discussed here, cell responses to environmental hypoxia during embryonic, fetal and adult myogenesis are regulated in a complex manner by interdependent pathways (Fig. 5). These synergistically regulate the balance between cell quiescence and proliferation, as well as between self-renewal and differentiation potential in an oxygen-dependent manner. Low oxygen levels contribute to promoting myoblast proliferation at the expense of their differentiation. As oxygen levels rise, cell proliferation is limited in favour of differentiation through the upregulation of myogenic genes. Continued analysis of the role of hypoxia in embryonic myogenesis and stem and/or progenitor cell behaviour, should reveal additional interactions between oxygen-sensitive regulators (such as HIFs) and the pathways that control proliferation and differentiation. Indeed, further efforts in defining more precisely the molecular interactions by which hypoxia influences myogenesis could ultimately contribute to the development of prevention and therapeutic strategies for muscle repair after damage induced by exercise or disease.

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