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Hypoxia Signaling Pathway in Stem Cell Regulation: Good and Evil

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Abstract

Purpose of Review—This review summarizes the role of hypoxia and hypoxia-inducible factors (HIFs) in the regulation of stem cell biology, specifically focusing on maintenance, differentiation, and stress responses in the context of several stem cell systems. Stem cells for different lineages/tissues reside in distinct niches, and are exposed to diverse oxygen concentrations. Recent studies have revealed the importance of the hypoxia signaling pathway for stem cell functions.

Recent Findings—Hypoxia and HIFs contribute to maintenance of embryonic stem cells, generation of induced pluripotent stem cells, functionality of hematopoietic stem cells, and survival of leukemia stem cells. Harvest and collection of mouse bone marrow and human cord blood cells in ambient air results in fewer hematopoietic stem cells recovered due to the phenomenon of Extra PHysiologic Oxygen Shock/Stress (EPHOSS).

Summary—Oxygen is an important factor in the stem cell microenvironment. Hypoxia signaling and HIFs play important roles in modeling cellular metabolism in both stem cells and niches to regulate stem cell biology, and represent an additional dimension that allows stem cells to maintain an undifferentiated status and multilineage differentiation potential.

Keywords

Hypoxia; HIF; Hematopoietic stem cells; Cancer stem cells; EPHOSS

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Introduction

Interactions between stem cells and their niche microenvironment are critical for stem cell self-renewal and protection of the stem cell pool [1–4]. Oxygen is a vital molecule in the stem cell microenvironment, serving as a metabolic substrate and signaling mediator [5]. While organs and tissues experience a considerably lower range of oxygen tension compared with the ambient oxygen levels (normoxia, 21%), measurements in stem cell niche revealed even lower oxygen tensions (Table 1) [6–15]. Low oxygen levels (hypoxia) are appreciated to maintain a slow-cycling proliferation property, reduced oxidative stress, and undifferentiated status in several stem cell populations [16–18].

The effects of hypoxia are primarily mediated by hypoxiainducible factors (HIFs) [19, 20]. HIFs are DNA-binding transcriptional factors of the basic-helix-loop-helix-Per-Arnt-Sim (bHLH-PAS) superfamily proteins and bind to hypoxia regulated elements (5′-RCGTG-3′, HREs) in the promoter and enhancer of hypoxia-inducible genes [21–23]. HIFs are heterodimers composed of an alpha subunit (HIF-α) and a beta (HIF-β) subunit, also known as aryl-hydrocarbon receptor nuclear translocator (ARNT) [20]. To date, three alpha subunits (HIF1A, HIF2A, HIF3A) and two beta subunits (ARNT, ARNT2) have been identified [23–26]. All of the HIF-α subunits contain an N-terminal bHLH domain for DNA binding, a PAS domain for heterodimerization, and an oxygen-dependent degradation (ODD) domain for regulation of protein stability. HIF1A is ubiquitously expressed, while expression of HIF2A and HIF3A is restricted to specific cell types [27]. ARNT and ARNT2 share 57% amino acid sequence identity and both contain bHLH and PAS domains [28, 29].

The HIF-α subunit is regulated by an oxygen-dependent proteolytic degradation, whereas the HIF-β subunit is constitutively expressed. Under ambient oxygen levels, HIF-α is hydroxylated on proline residues within the ODD domain by prolyl hydroxylases (PHD), which is recognized by von Hippel-Lindau (VHL) E3 ubiquitin ligase. VHL targets HIF-α for ubiquitin conjugation and proteasomal degradation (Fig. 1) [30–32]. Under hypoxia, when PHD activity is suppressed, HIF-α is stabilized and translocated to the nucleus where it dimerizes with ARNT to promote transcription of various hypoxia-inducible genes (Fig. 1) [33, 34]. HIF-α/ ARNT heterodimer can recruit histone acetyltransferases p300 and CBP to activate transcription of target genes [35, 36]. HIF-α activity is also regulated by additional protein modifications, such as phosphorylation [37], acetylation [38], and sumoylation [39].

While the roles of hypoxia signaling and HIFs in development have been reviewed by others, their effects on stem cells have received scant attention. In this review, we summarize recent advances on hypoxia signaling and HIFs with a focus on the regulation of the biology of several types of stem cells including pluripotent stem cells (PSCs), hematopoietic stem cells (HSCs), and cancer stem cells (CSCs).

Hypoxia and HIFs in Embryonic Stem Cells and Induced Pluripotent Stem Cells

Embryonic stem cells (ESCs) are capable of self-renewal and maintenance of pluripotency [40]. ESCs have been regarded as a potential replacement for tissues in regenerative

medicine due to their pluripotency. Studies have demonstrated the advantage of generating ESC lines under hypoxic conditions (5% O2) over ambient air, in terms of increased colonies with alkaline phosphatase activity and enhanced cell proliferation [41, 42]. Culturing cells in hypoxia reduced spontaneous differentiation and enhanced formation of embryoid bodies, and seemed to maintain the pluripotency of the cells [43, 44]. A marker for early differentiation (SSEA-1) was less expressed when human ESCs were cultured under hypoxia conditions [43]. Culturing human ESCs at hypoxia was correlated with increased expression of NANOG, OCT4, and SOX2, main regulators of the undifferentiated status of embryonic cells [45]. In addition, *HIF2A* depletion hampered proliferation and protein expressions of OCT4, SOX2, and NANOG in human ESCs [45]. Enhanced maintenance of self-renewal and pluripotency of human ESCs was observed when cocultured with human fetal liver stromal cells expressing HIF1A, suggesting that the function of HIF1A in the microenvironment is important to maintain the undifferentiated state of human ESCs [46].

Hypoxia plays a crucial role in determining the fate of the stem cells [47]. However, there are conflicting reports on the effect of hypoxia on ESC differentiation. Reports showed that hypoxia maintained self-renewal and prevented differentiation of ESCs [43]. In contrast, others revealed that hypoxia promoted differentiation of human ESCs into cardiomyocytes [48] and chondrocytes [49]. Hypoxia also promoted mouse ESCs to differentiate to neurons [50], endothelial cells, and hematopoietic stem cells [51]. These apparently controversial reports on the effect of hypoxia on differentiation may be explained by the stage of stemness at which the hypoxia was introduced, and the duration and degree of hypoxia. The mechanism through which HIF induces differentiation to different lineages has been revealing. Hypoxia primed ESCs to commit to the vascular lineage by suppressing Oct4 expression via direct binding of HIF1A in the Oct4 promoter region [52]. Another group reported that Hif1a induced mouse ESC commitment to arterial endothelia cells through upregulation of the transcription factor Etv2 and Notch1 signaling [53•]. Moreover, overexpression of Hif2a primed mouse ESCs to commit to cardiomyocytes by upregulating β-catenin [54]. Knocking out *HIF1A* by the CRSIPR-Cas9 system blocked transition from naive to primed human ESCs [55].

The groundbreaking finding by Yamanaka and colleagues demonstrated that somatic cells could be reprogrammed into induced pluripotent stem cells (iPSCs) by four transcription factors (4F; Oct4, Sox2, Klf4 and c-Myc) [56]. The generation of iPSCs was inefficient under normoxic conditions at ambient air (21% O2), and the reprogramming efficiency was significantly higher under mildly hypoxic conditions (5% O2) [57]. This finding suggests that the condition of hypoxia enhances stem cell generation and maintenance, consistent with observations in ESCs. One distinct characteristic of ESC and iPSC is their reliance on glycolytic metabolism, regardless of oxygen availability [58, 59]. Cellular bioenergetics are also extensively remodeled upon generation of iPSCs, from mitochondrial oxidative to glycolytic metabolism [60, 61]. Cells with disrupted HIF1A function are refractory to reprogramming [61]. Also, a small molecule activation of HIF1A upregulated HIF1A targets (PKM2 and PDK3), resulting in increased glycolysis and enhanced reprogramming efficiency [61]. One group continued to dissect the HIF regulatory role in metabolic switch and reprogramming [62]. They proposed that HIF1A and HIF2A were sufficient to induce metabolic switching during reprogramming. They distinguished between HIF1A and HIF2A

functions during reprogramming. Both were essential, but HIF2A applied its positive regulatory effect early in the process. Interestingly, prolonged stabilization of HIF2A impeded iPSC formation through TRAILinduced inhibition of caspase 3 [62]. Another report linked hypoxia, mitofusins (Mfn), and HIF1A with reprogramming [63]. Under hypoxic conditions, Mfn were downregulated and less expressed. In Mfn1/2 knockdown cells, HIF1A was stabilized, resulting in enhanced glycolytic metabolism and induced pluripotency [63].

Hypoxia Signaling and HIFs in Tissue-Specific Stem Cells, Using Hematopoietic Stem Cells as an Example

Each day, 100 billion new blood cells are produced by the human body. The continuous production of these cells depends on the presence of a rare population of HSCs [64–66]. HSCs are one of the best characterized adult stem cell types, and HSC transplantation is the only curative therapy for a variety of malignant and non-malignant blood diseases [67]. HSCs reside in a specialized bone marrow microenvironment, where they are adjacent to endothelial cells, osteoblasts, and stromal cells [68, 69]. Direct in vivo measurements revealed quite low oxygen tension in the BM of live mice with the lowest (1.3%) oxygen tension in deeper peri-sinusoidal regions [70••]. HSCs have a hypoxic profile including high levels of HIF1A protein expression [71], suggesting that hypoxia signaling plays an important role in HSC biology.

Genetic manipulations of mice have revealed the significance of HIFs in HSCs. Conditional deletion of *Hif1a* in the adult hematopoietic system resulted in loss of the quiescence state, and decreased repopulating capability of HSCs [72]. *Hif1a* null HSCs showed a metabolic shift from glycolysis to oxidative phosphorylation, with an increase in oxygen consumption rate and a decrease in anaerobic glycolysis [72, 73]. Consistently, the expression of pyruvate dehydrogenase kinases, which normally promotes glycolysis, was downregulated in *Hif1a* null HSCs [73]. Overexpression of pyruvate dehydrogenase kinases in *Hif1a*-null HSCs rescued the glycolysis phenotype and restored normal HSC activity. Conditional knockout of *Meis1* in HSCs resulted in decreased expression of *Hif1a* and *Hif2a*, and demonstrated a similar metabolic shift pattern (from glycolysis to mitochondria metabolism), loss of HSC quiescence, and impaired bone marrow engraftment after transplantation [74, 75]. It has been reported that deletion of *Hif2a* within the hematopoietic system had no impact on HSC function [76]; however, knockdown of *HIF2A* in human CD34+ cells (enriched for HSCs and their progenitor cells) resulted in impaired reconstitution capacity [77], suggesting a potential distinct role of HIF2A between mouse and human HSCs. Of note, *Hif2a*-null recipient mice failed to sustain hematopoiesis when transplanted with wild-type donor cells suggesting the importance and contribution of *Hif2a* in the HSC microenvironment for normal hematopoiesis [78].

Genetic knockouts on negative regulators of *Hif* resulted in enhanced hypoxia signaling and generated a significant phenotype in HSCs. Deletion of *Phd2*, a Hif prolyl hydroxylase, resulted in stabilization of both Hif1a and Hif2a [79]. Recipient mice transplanted with *Phd2* deficient bone marrow cells showed increased engraftment and donor reconstitution which

was reliant on Hif1a, indicating that Phd2 depletion and enhanced hypoxia signaling promote HSC proliferation after transplantation. Similarly, monoallelic loss of *Vhl*, an E3 ligase of Hif, enhanced cell cycle quiescence in HSCs and promoted engraftment after bone marrow transplantation [72]. Interestingly, biallelic loss of *Vhl* caused overstabilization of Hifs in HSCs and impaired transplantation capacity, possibly due to dysregulated cell cycle status and homing defects [72]. Furthermore, *Vhl* deletion in osteoblasts increased HSC and progenitor frequency, suggesting an important role of *Vhl* and *Hif*s in the HSC microenvironment [80]. In addition, conditional deletion of *Cited2*, another negative regulator of Hif1a, in the hematopoietic system resulted in loss of HSCs and bone marrow failure [81]. These results suggest that appropriate control of levels of Hifs in HSCs and their niche are essential for determining stem cell capacity.

HSC transplantation efficacy is a vital factor for successful donor engraftment during clinical therapy. Many efforts have been made to enhance human HSC transplantation efficacy via ex vivo expansion [82–86] or enhance homing [87•, 88, 89]. HIF1A has been reported to be involved in both of these efforts. Pharmacologic increase in HIF1A levels via prostaglandin E2 or dimethyloxalylglycine (DMOG) resulted in upregulation of surface CXCR4 expression, leading to enhanced HSC homing and engraftment [90]. In addition, HIF1A is essential for HSC mobilization induced by using G-CSF and AMD3100; pharmacologic stabilization of HIF1A by FG-4497 also enhanced mobilization of reconstituting HSCs [91]. NR-101, a novel c-MPL agonist, induced long-term accumulation of HIF1A protein and thus promoted ex vivo expansion of human cord blood HSCs, and enhanced their repopulating capacity [92].

Our laboratory recently reported that harvest, collection, and processing of mouse bone marrow and human cord blood HSCs under hypoxia (3% O2) resulted in 2–5-fold increases in the recovery of long-term repopulating HSCs compared with ambient air collection, through a phenomenon we named Bextra physiologic oxygen shock/stress^ (EPHOSS) [93••, 94]. EPHOSS is mediated by cyclophilin D and the mitochon-drial permeability transition pore (MPTP), with links to p53 and HIF1A. By modulating MPTP opening via cyclophilin D inhibition genetically or by using the small molecule inhibitor cyclosporine A, HSCs were protected from EPHOSS, resulting in increased collection of HSCs. This demonstrated that there are greater numbers of HSCs residing in bone marrow than previously reported, and suggesting that HSC transplantation could be improved if EPHOSS is suppressed by collecting and processing cells under hypoxia, or by using cyclosporine A or other modulators. Other means to suppress the phenomenon of EPHOSS for enhanced collection of HSCs are currently underway in our laboratory.

Hypoxia Signaling and HIFs in Cancer Stem Cells

Even though medical breakthroughs have advanced cancer care over the past decades, the high mortality rate due to disease recurrence remains one of the biggest challenges in the field. By definition, cancer stem cells (CSCs) are cells that have gained the abilities of self-renewal, as well as the capacity to reconstitute the heterogeneity of the tumor [95]. CSCs exist in several types of human cancers including blood malignancies [96] and solid tumors [97–99]. They are believed to be responsible for distant metastasis, tumor recurrence, and

therapy failure [100]. The expression of either HIF1A or HIF2A is positively correlated with poor prognosis in most of cancer types [101]. Recent evidence suggested that HIF1A plays a critical role for metabolic switch in cancer cells by regulating PKM2 [102]. While the role of hypoxia in selecting for solid tumor CSCs has been well-established [103, 104], the role that hypoxia and hypoxia-inducible factors play in the development and maintenance of leukemia stem cells (LSCs) is still a subject under debate [105]. The striking similarity between LSCs and HSCs in self-renewal capacity and the finding that HSCs reside in hypoxic niches in the bone marrow spurred interest in whether hypoxia also plays a critical role in LSC biology. Unfortunately, this poses an inevitable obstacle in targeting LSCs, without harming healthy HSCs.

A recent study demonstrated that the HIF1A–Notch pathway was responsible for sustaining CSCs self-renewal in two models of lymphoma and acute myeloid leukemia (AML), suggesting that this pathway may be able to be targeted to eliminate CSCs or at least to diminish the leukemia-initiating property of AML LSCs [106]. Notably, in the murine lymphoma model used (with insertional mutation of the *Epm2a* gene), the overexpression of Hif1a which is critical for CSC survival is interestingly hypoxia-independent because the degradation pathway of Hif1a by VHL is concurrently downregulated. In line with this, the same group also showed that echinomycin, a Hif1a inhibitor, when administered alone can prevent relapse in the $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ murine model of AML. The authors pointed out that this model, by not having the immunological barriers of xenogeneic grafts and carrying a spontaneous mutation found in a subgroup of AML patients, faithfully represented the human disease [106]. In addition to studies in AML, the roles of HIFs in progression and recurrence of PML-RARα-driven acute promyelocytic leukemia (APL) have been evaluated in two different human APL cell lines—APL NB4 and U937-PR9 [107]. Their microarray analysis of leukemic APL samples showed a HIFregulated gene profile significantly distinguishable between normal human promyelocytes and leukemic promyelocytes. In xenograft experiments, shRNA-induced inhibition of *HIF1A* reduced cell migration, colony forming ability, and prolonged mouse survival [107]. The results were confirmed in acute *HIF1A* inhibition with an RNA antagonist EZN2968, which showed even higher efficiency than chronic silencing of *HIF1A*. Complementary to these studies, the role of *HIF2A* in both normal human HSCs and progenitors, and AML cells was characterized [77]. Interestingly, expression of HIF2A in primary AML samples was not significantly different from normal bone marrow and varied between patients mostly because of diverse genetic abnormalities. However, similar to the findings with normal bone marrow, *HIF2A*knockdown AML cells showed defects in engraftment of leukemia including in a sample from a relapsed case; this defect resulted from endoplasmic reticulum stress-induced increase in apoptosis [77]. Thus, both HIF1A and HIF2A appear to be essential for protecting AML LSCs and maintenance of leukemia.

In contrast to the above findings, some skepticism has been posed on the therapeutic value of inhibition of HIFs in leukemia treatment [108]. Conditional knockout of *Hif1a* in three different well-characterized murine models of AML was used: *AML1-ETO9a* with no relation to *Hif1a*, and *Meis1* and *MLL* with direct signals for *Hif1a*. In all three models, the status of Hif1a expression did not appear to be critical for either the initiation or progression of leukemia. In fact, loss of *Hif1a* did not have any impact on self-renewal of LSCs and even

promoted their proliferation [108]. Adding to this study, others reported that HIF1A and HIF2A together acted as suppressors of AML development and had no significant impact on disease maintenance [109]. Loss of *Hif2a* unexpectedly enhanced LSC development and shortened AML latency in two murine models of AML (*Mll-AF9* and *Meis1/Hoxa9*) [109].

In addition to AML, the roles of hypoxia and HIFs in other types of LSCs have been investigated. Recent evidence suggested that HIF1A and hypoxia are crucial for the survival of chronic myeloid leukemia (CML) stem cells. HIF1A and its targets were overexpressed in BCRABL-expressing LSCs [110]. Using a BCR-ABL-driven CML murine model, it was observed that LSCs from *Hif1a* knockout mice had higher apoptotic rate and reduced colony formation, and were unable to propagate CML in secondary recipient. It was concluded that *Hif1a* is important for both development and maintenance of CML [110]. In agreement with these findings, another group also studied the role of hypoxia in CML but used primary human CML cells instead. They confirmed that hypoxia nurtured LSCs through upregulation of HIF1A even when BCR-ABL1 was effectively inhibited by imatinib [111]. They identified a profile of genes upregulated by hypoxia that are essential for cell survival in CML cells compared with healthy cord blood CD34$^+$ cells [111]. Similar results were noted in bortezomib-resistant CML LSCs using both the K562 cell line and primary patient CML cells [112]. While research in the past 5 years has focused mostly on CML and AML, a study looked at T cell acute lymphoblastic leukemia (T-ALL) [113]. Intriguingly, HIF1A was found to induce expression of β-catenin, and the Wnt-β-catenin pathway was confined to the LSC subpopulation. Furthermore, deletion of HIF1A or β-catenin significantly reduced the LSC frequency but not the viability of bulk tumor cells.

Conclusions

HIFs are primary mediators of the metabolic switch in the hypoxic environment. Genetic manipulation of HIFs and other hypoxia signaling components by gene knockout in mice yielded different phenotypes in various stem cell compartments as reviewed above. This seems to support the idea that hypoxia signaling is critical in maintenance of stem cell function. Physiological oxygen environment or hypoxia greatly enhanced establishment of ESC lines, iPSC reprogramming efficiency, and recovery of HSCs. It will be interesting to see if they also share similar mechanisms involved in EPHOSS, as mediated by cyclophilin D and the MPTP [93••].

Hypoxia and HIFs contribute to the development and survival maintenance of LSCs in a number of blood malignancies. However, as in the case of AML, there have been conflicting results between studies, possibly due to different experimental designs and technical approaches, and further complicated by the genetic heterogeneity of human AML disease. Another very important point and particularly relevant for this topic to be considered is that the cells studied should be collected and processed in hypoxia to avoid the effects of EPHOSS [93••]. As demonstrated by our laboratory, collection of HSCs at ambient O2 levels induced ROS production and altered cell function, and likely gene expression patterns; hence, it would seem to be counterproductive for a true understanding of LSCs to study the physiology and pathology of LSCs without maintaining these cells in their native hypoxic status. In other types of leukemia, future studies are warranted as well because the concept

of hypoxia in drugresistant LSCs is still emerging. Therefore, until a more thoroughly understanding of the role of HIFs in leukemia and LSCs is established, whether inhibition of HIFs promises therapeutic value will remain a question.

Acknowledgements

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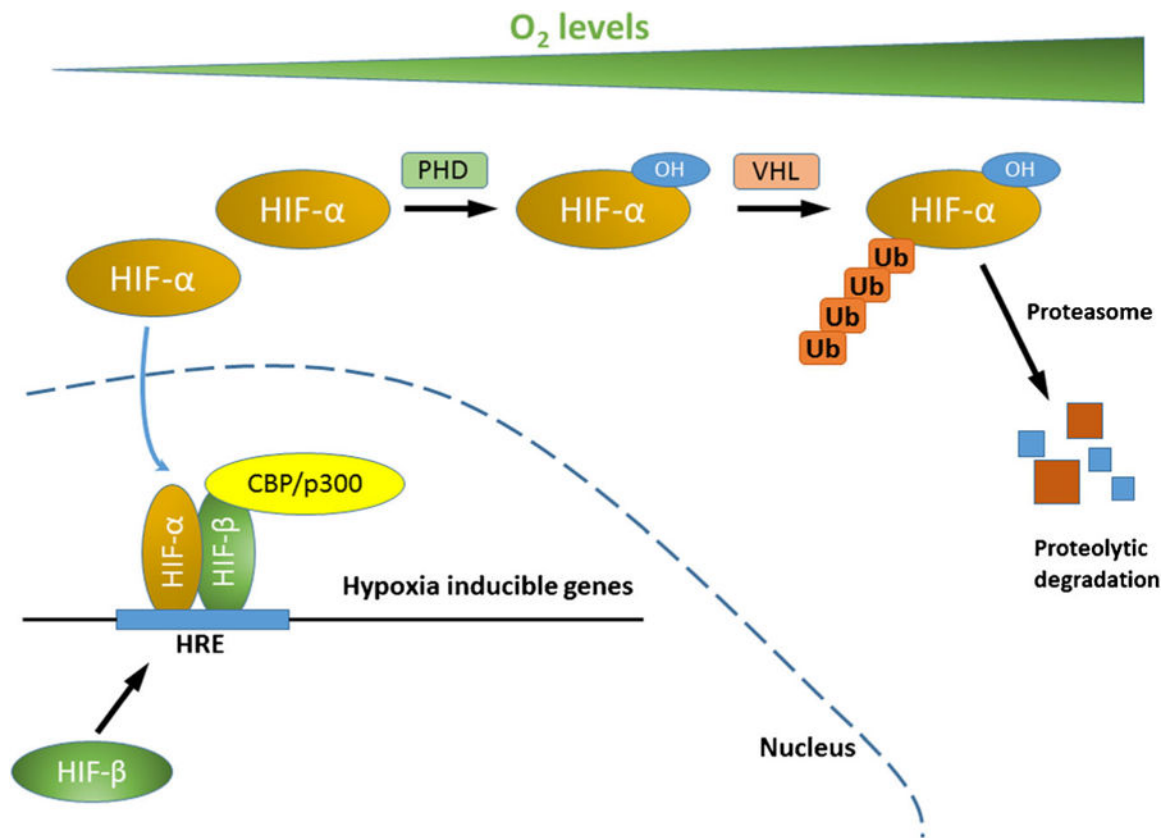
Fig. 1.
HIF mediated hypoxia signaling. Under normoxic oxygen tension, HIF-α is hydroxylated on proline residues by PHD, which is recognized by VHL E3 ubiquitin ligase. VHL targets HIF-α for polyubiquitin conjugation and proteasomal degradation. Under hypoxia, when PHD activity is suppressed, HIF-α is stabilized and translocated to the nucleus where it dimerizes with HIF-β. HIF-α/HIF-β heterodimer binds to HRE at the promoters of hypoxia-inducible genes, recruits histone acetyltransferases p300 and CBP to activate transcription of target genes. (HIF, hypoxia-inducible factor; PHD, prolyl hydroxylase; OH, hydroxyl group; VHL, von Hippel-Lindau; Ub, ubiquitin; HRE, hypoxia regulated elements; CBP, CREB binding protein)

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Table 1

Low oxygen levels in diverse stem cell compartments

Stem cell compartments	Oxygen level measurements	References
Neural stem cell niche	1%–8% O_2	[7, 8]
Embryonic stem cell niche	2%–8% O_2	[9]
Mesenchymal stem cell niche	2%–8% O_2	[10–12]
Hematopoietic stem cell niche	1%–6% O_2	[13]
Epidermal stem cell niche	2%–5% O_2	[14, 15]