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The Hypoxia Regulatory System in Hematopoietic Stem Cells

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1. Introduction

Stem cells localize to specific sites called 'niches' in various tissues, where they are preferentially maintained by growth factors from the environment. Mammalian bone marrow (BM) has been shown to be relatively hypoxic compared to other tissues, and primitive hematopoietic cells, including hematopoietic stem cells (HSCs), are thought to localize to the most hypoxic microenvironments in the BM. The hypoxic *ex vivo* culture of BM cells or primitive hematopoietic progenitors results in the maintenance of the primitive phenotype and cell cycle quiescence (Mohyeldin et al., 2010; Suda et al., 2011). *Ex vivo* culture of human HSCs under hypoxia also stabilizes hypoxia-inducible factor-1α (HIF-1α), a master transcriptional regulator of the cellular and systemic hypoxic response, and induces various downstream effectors of HIF-1α (Danet et al., 2003). However, the regulatory mechanisms and functional effects of BM hypoxia on HSCs *in vivo* have not been fully elucidated.

In the stem cell niche, HSCs are quiescent and show slow cell cycling. Various extracellular ligands, including CXCL12 (Sugiyama et al., 2006), angiopoietin-1 (Arai et al., 2004), and/or thrombopoietin (TPO) (Qian et al., 2007; Yoshihara et al., 2007), contribute to the quiescence of HSCs. Quiescent HSCs are maintained at a lower oxidative stress state to avoid their differentiation and exhaustion (Jang & Sharkis 2007). HIF-1α is a bHLH-PAS–type transcription factor (Semenza, 2007, 2009, 2010). Under normoxic conditions, prolyl residues in the HIF-1α oxygen-dependent degradation domain (ODD) are hydroxylated by HIF prolyl hydroxylases (PHDs). The hydroxylated ODD domain of HIF-1α protein is recognized by an E3 ubiquitin ligase, the von Hippel-Lindau protein (VHL). In the autosomal dominant hereditary disorder von Hippel Lindau disease, VHL is mutated, resulting in overstabilized HIF-1α protein by the impaired ubiquitin-proteasome pathway. Under hypoxic conditions, PHDs are inactivated and HIF-1α protein escapes degradation. Several niche factors, such as thrombopoietin (TPO) (Kirito et al., 2005) and stem cell factor (SCF) (Pedersen et al., 2008), also stabilize HIF-1α protein in hematopoietic cells even under normoxic conditions.

Stabilized HIF-1α protein forms a heterodimeric transcriptional complex with the oxygen-independent subunit HIF-1β, translocates to the nucleus, and directly binds hypoxia-responsive elements found in the promoter regions of numerous downstream regulators, thereby activating their transcription. HIF-1β is reportedly required for hematopoietic cell

generation during ontogeny. However, a detailed analysis of the contribution of HIF-1α to the maintenance of adult HSCs has not yet been reported.

We analyzed HSCs in HIF-1α– and VHL-deficient mice and found that the cellular pool and cell cycle status of HSCs were regulated by the HIF-1α level (Takubo et al., 2010). Our analysis revealed that the regulation of the HIF-1α dose is critical for HSC maintenance in the hypoxic niche microenvironment of the BM. The critical role for HIF-1α in HSC cell cycle regulation broadens the involvement of oxygen status in the stem cell niche. It also implies a novel strategy for maintaining and expanding HSC resources based on cellular oxygen metabolism reprogramming, including the modulation of HSC quiescence through the oxygenation status of HIF-1α.

2. Quiescence of hematopoietic stem cells

Somatic stem cells contribute to tissue homeostasis throughout life (Suda et al., 2011). Because proliferation will induce senescence, the proper maintenance of stem cells without senescence is mandatory. There are two states for tissue stem cells in terms of the cell cycle. One is the quiescent state. Stem cells in the quiescent state are out of the active cell cycle (S/G2/M phase) and in the G0 phase. The other is the cycling state. Cycling stem cells actively reproduce themselves (self-renewal) to generate progeny. Cycling cells are in the non-G0 phase of the cell cycle. Quiescence is thought to be an effective strategy for stem cells to avoid various forms of cytotoxic damage. If stem cells lost quiescence, they would become susceptible to intrinsic and extrinsic stresses.

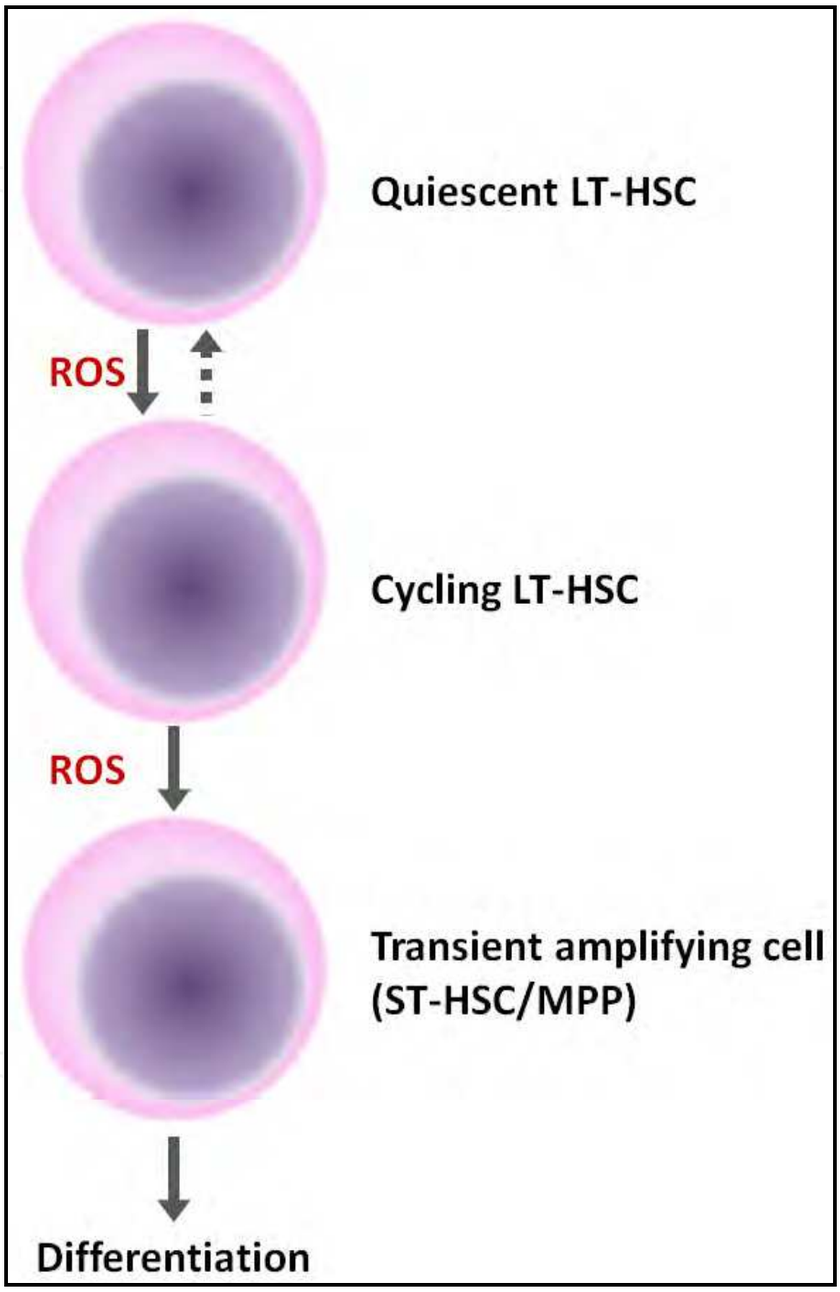
Mammalian HSCs are included in the heterogeneous population of lineage marker$^-$, Sca-1$^+$, and c-Kit$^+$ (LSK) cells. LSK cells are a mixture of progenitors and HSCs. Within the LSK population, CD34$^-$CD150^{hi}CD48$^-$CD41$^-$Flt3$^-$ cells, as well as side population (SP) cells (Osawa et al, 1996; Kiel et al, 2005; Goodell et al, 1996), are quiescent.

Measurement of the cell cycle in HSCs by the staining of DNA with Hoechst 33342, DAPI, and/or anti-Ki67 indicates that more than 70% of highly purified HSC (CD34$^-$CD48$^-$CD150^{hi} LSK) are in the G0 phase, whereas less than 10% of CD34$^+$ LSK cells (differentiated progenitors) are in the quiescent phase (Wilson et al, 2008).

Slow-cycling HSCs have long-term (LT) reconstitution activity when they are transplanted into lethally irradiated recipient mice. In contrast, actively cycling HSCs and progenitors exhibit only short-term (ST) reconstitution activity and only maintain hematopoiesis for 3–4 months. Thus, the former are termed "LT-HSC(s)" and the latter are "ST-HSC(s)". LT-HSCs produce ST-HSCs, multipotent progenitors (MPPs), lineage-restricted progenitors, and terminally differentiated hematopoietic cells including erythrocytes, platelets, lymphocytes, granulocytes, and macrophages (Figure 1).

Because slow-cycling stem cells are not in the S or M phase of the cell cycle, they are more resistant to cytotoxic agents such as ultraviolet (UV) light, ionizing radiation and chemicals, compared with actively cycling cells. Recent reports indicate that quiescent stem cell fractions are present in several tissues. For example, in the hair follicle, cell cycle progression of stem cells in bulge regions is suppressed by Wnt inhibitors (Fuchs and Horsley, 2011). In contrast, stem cells in the murine intestinal and gastric epithelia divide every 24 hours

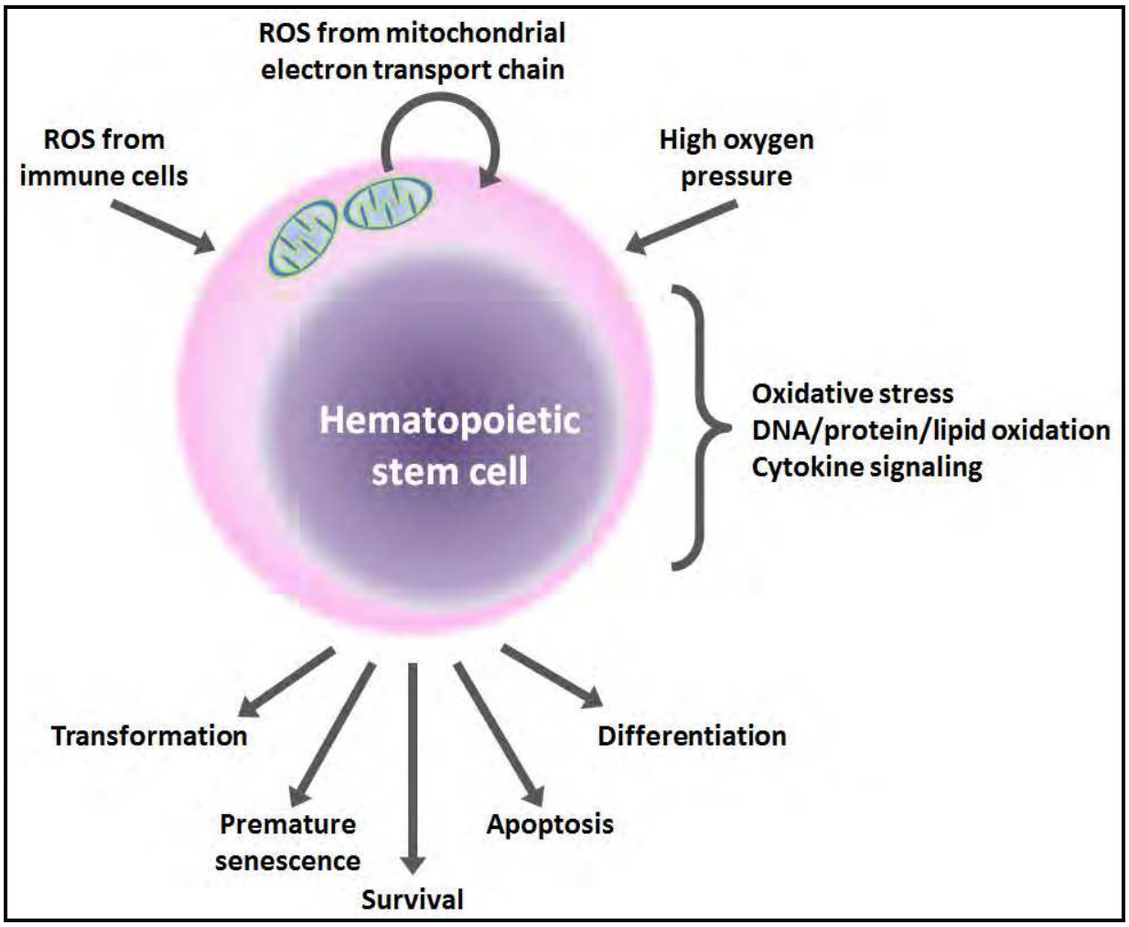
(Snippert and Clevers, 2011). Therefore, quiescence itself is not the only strategy for the long-term maintenance of stem cells.



Life-long hematopoiesis is maintained by long-term (LT)-HSCs and their progeny. LT-HSCs have a two cell cycle states: a quiescent state (G0 phase) and a cycling state (non-G0; i.e., G1/S/G2/M phase). LT-HSCs in the former state are resistant to various cytotoxic stresses. Reactive oxygen species (ROS) change the cell cycle state of LT-HSCs from quiescent to cycling. Cycling LT-HSCs are also promoted to differentiate into short-term (ST)-HSCs and multipotent progenitors (MPPs). These differentiated progenitors actively produce various terminally differentiated hematopoietic cells.

Fig. 1. Quiescent and cycling hematopoietic stem cells (HSCs)

One important regulator for the quiescence of HSCs is reactive oxygen species (ROS). ROS are an intrinsic and extrinsic stress for HSCs (Figure 2). Intrinsically, ROS are mainly produced by mitochondria, the energy factory of the cell, as a by-product of the electron transport chain. Because anaerobic energy metabolism in mitochondria utilizes oxygen to generate ATP, oxygen-rich conditions produce intracellular ROS in HSCs. In addition, various immune cells utilize oxygen to generate ROS as an anti-microbial agent. ROS have favourable and unfavourable effects on HSCs. ROS are a signal transducer for essential cytokine signalling in HSCs (Sattler et al., 1999). However, excessive or prolonged ROS exposure is detrimental to HSCs (Naka et al., 2008). Aberrant exposure to ROS induces senescence, apoptosis, or the accumulation of DNA damage in HSCs. These damaged cells are dysfunctional and a potential source for leukemic transformation. Therefore, it is reasonable to hypothesize that HSCs reside in a hypoxic microenvironment.



HSCs are exposed to ROS from various sources, including endogenous mitochondria and adjacent immune cells. High O2 pressure in the microenvironment also promotes ROS generation. This ROS burden results in the oxidation of DNA, protein, and lipid in HSCs. Also, the appropriate dose of ROS mediates cytokine signalling in HSCs. These balances determine the fate of HSCs: survival, premature senescence, apoptosis, differentiation, or malignant transformation.

Fig. 2. Intrinsic and extrinsic oxidative stresses and HSCs

3. Hypoxic nature of bone marrow

Although molecular oxygen is critically important for living organisms, HSCs are susceptible to reactive oxygen species or oxidative stresses that are derived from molecular oxygen. To maintain life-long hematopoiesis, it is reasonable for HSCs to avoid high-oxygen conditions. Although classical observations and theoretical studies supported these views, experimental evidence has only been recently provided.

Classically, bone marrow has been thought to be hypoxic. Recently, its exact nature and dynamic regulation were studied. This section will summarize the classical and recent studies related to the functional anatomy of bone marrow oxygenation.

Genetic studies have postulated that LT-HSCs reside primarily in the endosteal zone of the bone marrow (BM) (Calvi et al, 2003; Zhang J, 2003; Arai et al, 2004). Vascular organization around the endosteal zone is unique (Draenert and Draenert, 1980). Nutrient arteries penetrate the cortical bone, enter the medullary canal, and then proceed in a spiral pattern into the metaphyseal region of the bone marrow. The blood in arterial capillaries drains into sinusoids, which are fenestrated and loosely organized.

As a result, hematopoietic cells can easily move across the sinusoidal endothelium. Accordingly, the perfusion of the BM is limited and the partial oxygen pressure (PO2) in the endosteal region is very low.

In addition to hypoperfusion, the BM is tightly packed with blood cells. Oxygen consumption by hematopoietic cells is relatively high, and a simulation of O2 diffusion in the bone marrow suggested that the PO2 is decreased 10-fold at a distance of several cells from the nearest capillary (Chow et al, 2001). The average PO2 in the BM is approximately 55 mmHg and the mean O2 saturation is 87.5% (Harrison et al, 2002). Thus, based on this simulation study, HSCs may well reside in a severely hypoxic environment.

In support of this idea, it has also been reported that murine HSCs live in a hypoxic BM niche. By administering a perfusion tracer into mice, one group found that HSCs accumulated in a hypoperfusion cellular fraction in the BM (Parmar et al, 2007). These hypoperfused cells retained pimonidazole, a probe that selectively binds and forms adducts with protein thiol groups in a hypoxic environment. Administration of a toxin selective for hypoxic cells (tirapazamine) resulted in the depletion of HSCs *in vivo*. It was also shown that LT-HSCs are positive for pimonidazole in mice (Takubo et al, 2010). Moreover, human cord blood stem cells transplanted into super-immunodeficient NOD/scid/IL-2Rγ (NOG) mice homed to the BM niche and became both hypoxic and quiescent after BM transplantation (Shima et al, 2010).

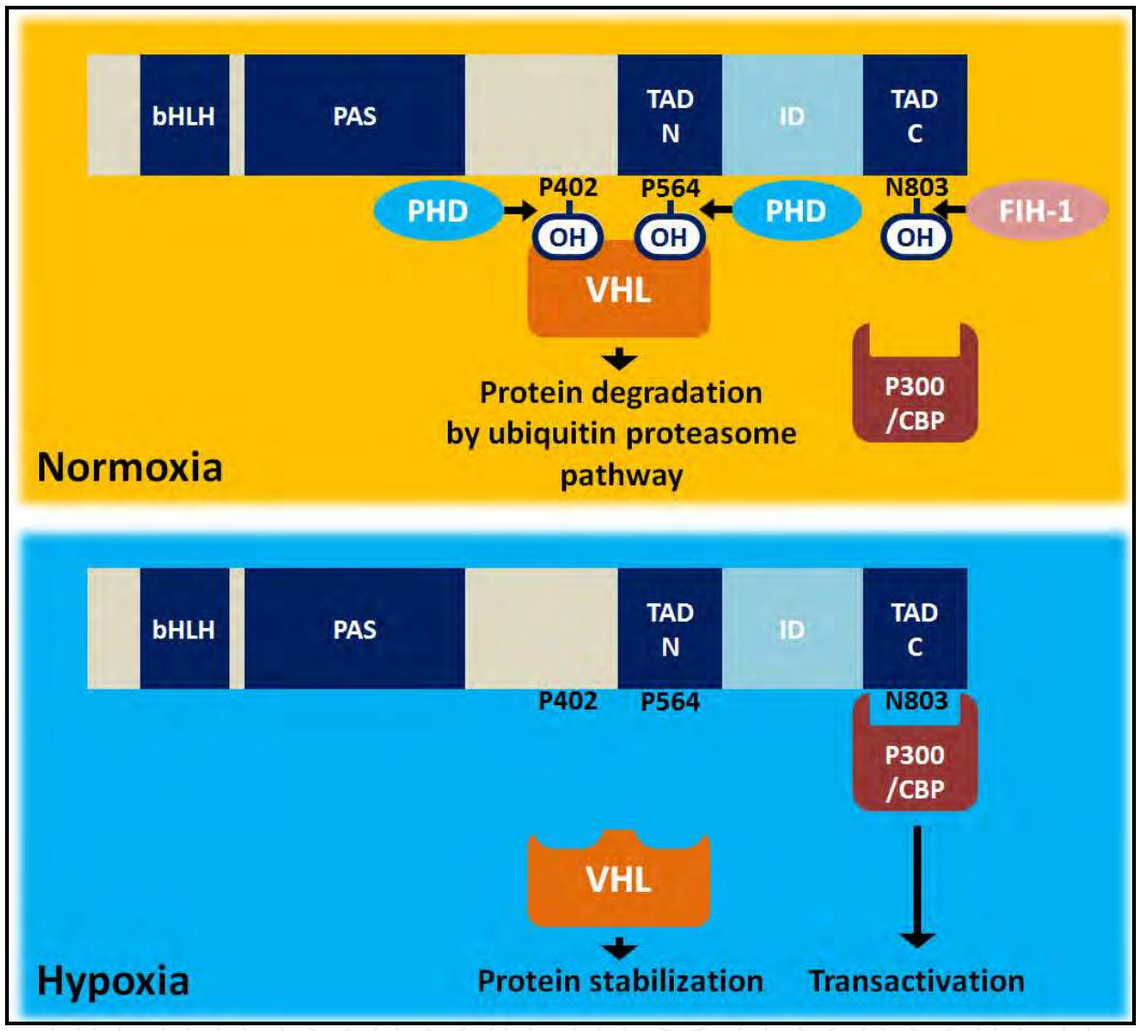
Collectively, these findings suggest the hypoxic nature of HSCs. The hypoxic character of LT-HSCs is potentially determined by their position within the BM. However, in contrast to the simple O2 gradient model for the BM hypoxic niche, immunohistochemical observation of a two-dimensional segment of the murine BM suggests that 60% of LT-HSCs localize closely to BM endothelial cells (Kiel et al, 2005; Sugiyama et al, 2006). These findings do not fit the simple O2 gradient model for the hypoxic status of HSCs in the niche. However, as noted above, the vasculature in the niche near the endosteal zone of the BM may perfuse the bone marrow very poorly. Four-dimensional tracking (time-

lapse and three-dimensional observation with a multi-photon microscope) of single LT-HSCs in the BM has shed light on this paradox. Real-time tracking of murine BM revealed that HSCs gradually move away from bone marrow blood vessels and then detach from them and translocate to the osteoblastic zone of the BM after transplantation (Lo Celso et al, 2009; Xie et al, 2009). Based on these observations, it is possible that subpopulations of HSCs residing in different specific locations have different oxygenation statuses.

In parallel with the hypoxic microenvironment for HSCs *in vivo*, hypoxic culture phenotypically and functionally sustained HSCs more effectively than normoxic culture (20% oxygen). Also, hypoxic culture enhances the colony-forming ability (progenitor ability) and transplantation capacity (HSC capacity) of cultured BM cells or isolated HSCs (Cipolleschi et al, 1993; Danet et al, 2003; Ivanovic et al, 2004). Hypoxic treatment also induces cell cycle quiescence in cultured HSC (Hermitte et al, 2006; Shima et al, 2010). Quiescent HSCs are defined by a high amount of efflux of the DNA-binding dye Hoechst 33342 from the cytosol (Goodell et al, 1996). These cells are called "side population (SP)" cells due to their specific staining pattern by flow cytometric analysis. Hypoxic treatment also sustains the SP phenotype in HSC *in vitro* (Krishnamurthy et al, 2004). Exclusion of Hoechst dye from the HSC cytosol is supported by Bcrp1/ABCG2, an ATP-dependent transporter, at the plasma membrane. When HSCs were cultured under hypoxic conditions, mRNA expression of Bcrp1/ABCG2 was significantly increased and the number of SP cells was also increased as compared to HSCs cultured at normoxia. Interestingly, because Bcrp1-/- mice show no significant defect in hematopoiesis (Zhou et al, 2001), the functional role of Bcrp1 in HSCs is still uncharacterized.

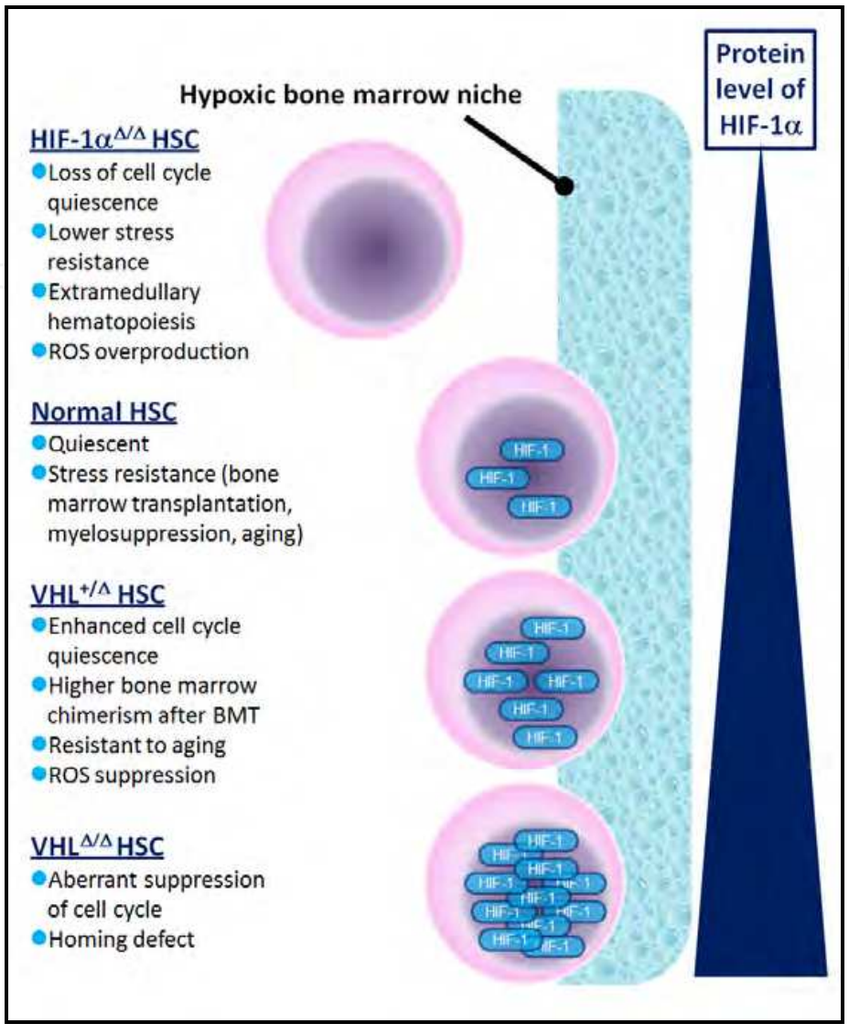
4. Hypoxia response system in HSCs

Cells sense, respond, and adapt to hypoxia using hypoxia-responsive regulatory pathways. HSCs utilize the same hypoxia response pathways as a number of other cell types. A central component of these pathways is hypoxia-inducible factor-1 (HIF-1), a transcription factor that is essential for cellular and systemic responses to a low oxygen microenvironment (Semenza, 2010) (Figure 3). HIF-1 is a heterodimeric transcription factor consisting of the oxygen-dependent HIF-1α subunit and an oxygen-independent HIF-1β subunit (Wang and Semenza, 1995). HIF-1α is hydroxylated at proline (Pro) 402 and/or 564 in the oxygen-dependent degradation (ODD) domain under normoxic conditions (Kaelin and Ratcliffe, 2008). HIF-1α is hydroxylated by three prolyl hydroxylases (PHD1–3) which require molecular oxygen, Fe2+, 2-oxoglutarate, and ascorbic acid for their full enzymatic activity (Epstein et al, 2001). Prolyl-hydroxylated HIF-1α protein is recognized by the von Hippel-Lindau (VHL) tumor suppressor protein, which recruits the Elongin C/Elongin B/Cullin2/E3 ubiquitin ligase complex. As a result, prolyl-hydroxylated HIF-1α protein is ubiquitinated and degraded by the proteasome. Under a hypoxic environment, prolyl hydroxylases lose their enzymatic activity. Thus, prolyl hydroxylation of HIF-1α is suppressed, and HIF-1α protein is stabilized without degradation (Kaelin and Ratcliffe, 2008). HIF-1 heterodimers (HIF-1α:HIF-1β) are recruited and bind to hypoxia response elements (HREs) in various target genes and activate transcription programs (Semenza, 2010).



The diagrams represent the regulation of HIF-1α protein and interacting factors under different oxygen conditions. HIF-1α is a substrate for both prolyl and asparaginyl hydroxylases. Under normoxia, proline and asparagine residues are hydroxylated. These modifications regulate the stability and transcriptional activity of HIF-1α. bHLH, basic-helix-loop-helix domain; PAS, Per-ARNT-Sim domain; TAD-N, transactivation domain N-terminal; ID, inhibitory domain; TAD-C, C-terminal transactivation domain; PHD, prolyl hydroxylase domain-containing protein; and FIH-1, factor-inhibiting HIF-1.

Fig. 3. Regulation of hypoxia-inducible factor-1α (HIF-1α)



Scheme of biological outcomes of different HIF-1α protein levels in HSCs. This is achieved by HIF-1α or VHL deletion in HSCs using knockout mouse models. Normal HSCs (the second from the top) stabilize HIF-1α, which maintains cell cycle quiescence at the hypoxic bone marrow niche in the endosteum. Preferential stabilization of HIF-1α was observed in HSCs under hypoxia. HIF-1$\alpha^{\Delta/\Delta}$ HSCs (top) lose cell cycle quiescence and stress resistance against transplantation, chemotherapeutic agents, and aging. In addition, HIF-1$\alpha^{\Delta/\Delta}$ HSCs leave the bone marrow niche and drive extramedullary hematopoiesis in the spleen. Production of ROS is accelerated in HIF-1$\alpha^{\Delta/\Delta}$ HSCs. Heterozygous deletion of VHL results in a slight increase in HIF-1α protein. Under these conditions, cell cycle quiescence in HSCs is enhanced. The VHL$^{+/\Delta}$ HSC (the second from the bottom) is resistant to transplantation and aging. ROS production is also suppressed in VHL$^{+/\Delta}$ HSCs. The homozygous VHL mutant (VHL$^{\Delta/\Delta}$) HSC has a maximal dose of HIF-1α protein. In contrast to heterozygous VHL mutant HSCs, VHL$^{\Delta/\Delta}$ HSCs completely lost stem cell capacity potentially due to aberrant suppression of the cell cycle and/or homing capacity to the niche. This defect is HIF-1α–dependent because the co-deletion of HIF-1α in homozygous VHL-deficient hematopoietic cell rescued the defect. Thus, the precise regulation of HIF-1α levels coordinates stem cell proliferation and differentiation. Recently, it has been reported that vascular endothelial growth factor, heat shock proteins, and GRP78 and its ligand Cripto regulate HSC quiescence and maintain HSCs in hypoxia as downstream factors of HIF-1α (Rehn et al, 2011; Miharada et al, 2011).

Fig. 4. Features of HIF-1α or VHL knockout HSCs

HIF-1α mRNA and protein are highly expressed in LT-HSCs (Takubo et al, 2010; Simsek et al, 2010) (Figure 4). HSCs derived from conditional HIF-1α knockout (HIF-$1\alpha^{\Delta/\Delta}$) mice have a defective capacity for marrow reconstitution during serial BM transplantation (Takubo et al., 2010). HIF-$1\alpha^{\Delta/\Delta}$ LT-HSCs lost cell cycle quiescence, entered the cell cycle from G0 phase, proliferated, and showed reduced tolerance to stresses such as 5-fluorouracil administration or aging. These studies suggest that HIF-1α plays an essential role in the regulation of HSC quiescence and stress resistance *in vivo*. In addition to these HIF-1α loss-of-function studies, conditional deletion of the VHL gene in hematopoietic cells was performed as a HIF-1α gain-of-function experiment. Analysis of VHL mutant hematopoietic cells revealed that the functional properties of LT-HSCs and progenitors are differentially influenced by HIF-1α. HIF-1α protein levels are elevated in either biallelic ($VHL^{\Delta/\Delta}$) or monoallelic ($VHL^{+/\Delta}$) conditional knockout hematopoietic cells. For example, only a minor population of normal hematopoietic progenitors ($CD34^+$ LSK cells) are in a quiescent state. In clear contrast, the proportion of $VHL^{+/\Delta}$ hematopoietic progenitors in the quiescent phase is significantly higher. At steady state, HIF-1α protein levels are not high in hematopoietic progenitors, and forced stabilization of HIF-1α protein through monoallelic VHL deletion induces $VHL^{+/\Delta}$ $CD34^+$ LSK progenitors to exit the cell cycle and maintains them in the G0 phase. Severe suppression of cell cycling and transplantation capacity is restored in HIF-$1\alpha^{\Delta/\Delta}$:$VHL^{\Delta/\Delta}$ doubly mutated HSCs. The decreased frequency of LT-HSCs seen in $VHL^{\Delta/\Delta}$ mice is rescued by the co-deletion of the HIF-1α gene *in vivo*. Also, long-term *in vitro* exposure of LT-HSCs to a PHD inhibitor (dimethyloxalylglycine; DMOG), which stabilizes HIF-1α even under normoxic conditions, attenuates stem cell ability especially during BM transplantation (Eliasson P et al, 2010).

Collectively, these results provide evidence that there is an optimal HIF-1α protein level for HSC maintenance. HIF-1α is required for stress resistance and long-term maintenance of HSCs, and within an appropriate range, moderate increases of HIF-1α (to the level caused by VHL heterozygous deletion) are trophic for HSCs through the induction of quiescence. However, aberrantly high HIF-1α levels are also harmful to HSCs and lead to a loss of stem cell capacity and the exhaustion of the HSC pool. Homozygous deletion of VHL results in a severe suppression of the cell cycle and a homing defect during transplantation.

HIF-1α not only acts in the HSC system but also plays an important role in neural stem cells (NSCs) under hypoxic conditions. In this type of cell, HIF-1α induces the activation of the Wnt/β-catenin signalling pathway through the upregulation of β-catenin and the expression of the downstream transcription factors lymphoid enhancer-binding factor 1 and T-cell factor 1 (Mazumdar J et al, 2010). Wnt/β-catenin activity was closely correlated with hypoxic status in the subgranular zone of the hippocampus, which is one of the niches for NSCs. Loss of HIF-1α in NSCs resulted in a defective Wnt-dependent hippocampal neurogenic niche capacity. As a result, NSC proliferation and differentiation, and the production of new neurons, were attenuated. Interestingly, the biological effects of HIF-1α on NSCs (cell cycle promotion) are clearly different from those seen in HSCs (cell cycle quiescence). It will be important to dissect how these different lineage stem cell systems utilize the same protein (HIF-1α) to sustain themselves using different downstream molecular machinery and biological events. It is also of interest to investigate embryonic

HSCs, which actively proliferate in hypoxic conditions, because HIF-1α may support HSC proliferation in that stage. In addition, HIF-1α protein has been reported to inhibit Wnt/β-catenin activity in cancer cells (Kaidi A et al, 2007), suggesting that the interaction of the HIF-1α and Wnt/β-catenin pathway in stem/progenitor cells may differ from that of more differentiated or transformed cell types.

5. Conclusion

In this chapter, I have summarized our current knowledge regarding the hypoxia response and oxygen metabolism in HSCs at the BM niche. These studies open novel fields in stem cell biology. The invisible niche factor, oxygen, is usually essential because mitochondria utilize it for the energy production. However, molecular oxygen is a source of ROS during mitochondrial metabolism. Because an excessive dose of ROS can be damaging to HSC, escape from oxygen (in the hypoxic niche) is a reasonable strategy for the long-term maintenance of HSCs *in vivo*. Adult HSCs are quiescent and contain few mitochondria, whereas hematopoietic progenitor cells actively proliferate and contain many mitochondria. Thus, stem cells and progenitors have distinct metabolic states, and the transition from stem to progenitor cell may correspond to a critical metabolic change, namely from glycolysis to oxidative phosphorylation. Slow cell cycling or long-term quiescence is common in adult tissue stem cells. Dormancy in the cell cycle may be a crucial mechanism for the stress resistance of normal and leukemic stem cells.

Further investigation of oxygen metabolism in tissue stem cells will result in more effective maintenance, expansion, and manipulation of various somatic stem cells *ex vivo* and *in vivo*, maximizing the potential of therapeutic strategies using stem cells in regenerative medicine. Also, an understanding of oxygen homeostasis in HSCs is essential for understanding senescence at the stem cell level as well as therapeutic targeting against leukemic stem cells.

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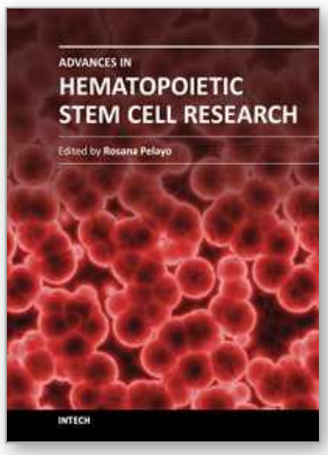

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This book provides a comprehensive overview in our understanding of the biology and therapeutic potential of hematopoietic stem cells, and is aimed at those engaged in stem cell research: undergraduate and postgraduate science students, investigators and clinicians. Starting from fundamental principles in hematopoiesis, Advances in Hematopoietic Stem Cell Research assemble a wealth of information relevant to central mechanisms that may regulate differentiation, and expansion of hematopoietic stem cells in normal conditions and during disease.

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