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REVIEW

To breathe or not to breathe: the haematopoietic stem/progenitor cells dilemma

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Adult haematopoietic stem/progenitor cells (HSPCs) constitute the lifespan reserve for the generation of all the cellular lineages in the blood. Although massive progress in identifying the cluster of master genes controlling self-renewal and multipotency has been achieved in the past decade, some aspects of the physiology of HSPCs still need to be clarified. In particular, there is growing interest in the metabolic profile of HSPCs in view of their emerging role as determinants of cell fate. Indeed, stem cells and progenitors have distinct metabolic profiles, and the transition from stem to progenitor cell corresponds to a critical metabolic change, from glycolysis to oxidative phosphorylation. In this review, we summarize evidence, reported in the literature and provided by our group, highlighting the peculiar ability of HSPCs to adapt their mitochondrial oxidative/bioenergetic metabolism to survive in the hypoxic microenvironment of the endoblastic niche and to exploit redox signalling in controlling the balance between quiescence versus active cycling and differentiation. Especial prominence is given to the interplay between hypoxia inducible factor-1, globins and NADPH oxidases in managing the mitochondrial dioxygen-related metabolism and biogenesis in HSPCs under different ambient conditions. A mechanistic model is proposed whereby 'mitochondrial differentiation' is a prerequisite in uncommitted stem cells, paving the way for growth/differentiation factor-dependent processes. Advancing the understanding of stem cell metabolism will, hopefully, help to (i) improve efforts to maintain, expand and manipulate HSPCs *ex vivo* and realize their potential therapeutic benefits in regenerative medicine; (ii) reprogramme somatic cells to generate stem cells; and (iii) eliminate, selectively, malignant stem cells.

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Abbreviations
AMPK, AMP-activated PK; BM, bone marrow; FAO, fatty acid oxidation; HIF-1, hypoxia inducible factor-1; HRE, hypoxia response elements; HSC, haematopoietic stem cell; HSPC, haematopoietic stem/progenitor cell; Lkb1, liver kinase B1; LT-HSC, long-term HSC; OXPHOS, mitochondrial oxidative phosphorylation; NOX, NADPH oxidase; PGC-1α, PPARγ co-activator 1α ; PML, promyelocytic leukaemia; RC, respiratory chain

Introduction

The mammalian blood system comprises an array of cell types, including erythrocytes, myeloid cells, megakaryocytes and platelets, lymphocytes, natural killer cells, dendritic cells and mast cells. As diverse as these cells are, they all originate from haematopoietic stem cells (HSCs), which are a limited pool of immature progenitors residing in the bone marrow (BM). A reservoir of long-term HSCs (LT-HSCs) lies at the very top of the cellular hierarchy. LT-HSCs guarantee a continuous supply of blood cells throughout an individual's lifetime due to their potential to self-renew (give rise to identical daughter cells) and differentiate. Pools of stem and progenitor cells with decreasing self-renewal potential are downstream of

LT-HSCs: the short-term HSCs and multipotent progenitors, which retain full differentiation capacity, and the lineage-restricted progenitors.

These downstream progenitors are the real workhorses of the haematopoietic system, as they divide rapidly and generate a large number of differentiated progeny. In order to guarantee blood homeostasis, the system is tightly regulated but also highly resilient and is capable of modulating the production of specific progeny in response to bleeding, infections or environmental insults. This resilience is the result of two opposing forces – one sustaining HSC dormancy, or quiescence, which is critical to maintain a reservoir of stem cells, and the other activating proliferation and differentiation. Disrupting this balance can have a variety of pathological consequences, such as BM failure or haematological malignancy. Thus, it is important to understand the forces that regulate haematopoietic stem and progenitor cell (HSPC) function (Orkin and Zon, 2008).

Expression of cell surface markers on human haematopoietic cells has provided a method for purifying and characterizing the hierarchical organization of cell subsets with distinct biological functions. Although the identification of marker(s), which specifically individualize(s) the primitive multipotent HSC is still lacking and is the object of research and controversy (Schroeder, 2010), it is generally accepted that the $CD34^+/CD38^-$/lineage commitment marker $(Lin)^-$ is a phenotype signature consistent with most, if not all, of the haematopoietic activity in BM (Srour *et al.*, 1991; Bhatia *et al.*, 1997; Seita and Weissman, 2010). Successful engraftment of baboons with BM highly enriched for $CD34^+$ cells led to the widespread use of CD34-enriched populations in human transplantation (Berenson *et al.*, 1988). In general, patients transplanted with marrow or mobilized peripheral blood, enriched with CD34 cells [typically induced by granulocyte colony-stimulating factor (G-CSF) pretreatment of the donor] engraft rapidly and seem to have fewer transplant-related complications (Civin *et al.*, 1996).

However, the HSPCs' $CD34^+/CD38^-/Lin^-$ probably constitutes a heterogeneous pool of cell subsets comprising active cycling and self-renewing stem cells and an array of proliferating (but not self-renewing) early progenitors and lineage (pre)-committed haematopoietic cells (Sakabe *et al.*, 1997; Schroeder, 2010; Koutna *et al.*, 2011). Importantly, the higher the expression of CD34 in a given HSPC subset is, the higher its efficacy both in an *in vivo* long-term re-populating assay (Krause *et al.*, 1996) and in clinical BM transplantation outcome (Beksac and Preffer, 2012). This can be explained either as the occurrence of a specific stem cell subpopulation within the $CD34^{high}$-HSPC pool or as a higher propensity of $CD34^{high}$-HSPC to reverse their pre-commitment thereby recovering features of multipotent stem cells (Dooner *et al.*, 2008; Knaän-Shanzer *et al.*, 2008; Puca *et al.*, 2012).

Mitochondrial and metabolic control of the HSPC fate

Transcriptome, epigenome and proteome studies are providing important insights into stem cell biology (Macarthur *et al.*, 2009), indicating that specific metabolic properties are required to maintain self-renewal and the multipotent differentiation capacities of HSCs (Suda *et al.*, 2011; Folmes *et al.*, 2012). It is a common belief, recently supported by experimental evidence, that quiescent HSCs mainly rely on anaerobic glycolysis for energy production (Simsek *et al.*, 2010; Takubo *et al.*, 2013). This hypothesis is based on the notion that primitive HSCs reside in a specific BM tissue environment close to the endosteum where the limited oxygen supply makes this region hypoxic (this point will be discussed in more depth in the next paragraph) and therefore unsuited for mitochondrial oxidative phosphorylation (OXPHOS) (Eliasson and Jönsson, 2010). The OXPHOS pathway is made up of an inner mitochondrial membrane-located respiratory chain (RC), which transfers reducing equivalents along a set of respiratory complexes (I-IV) to O_2. The free energy, as such made available, is converted to an electrochemical transmembrane potential, which drives the ATP synthesis, by the FoF1-ATP-synthase complex (Papa *et al.*, 2012). The cytochrome *c* oxidase is the RC complex responsible for the final transfer of reducing equivalents to O_2, which is converted into two H_2O molecules. The estimated K_M for O_2 of cytochrome *c* oxidase is in the submicromolar range (Wilson *et al.*, 1988; Scandurra and Gnaiger, 2010) (1.0 μM of dissolved O_2 in water is in equilibrium with some 0.1% of pO_2). This value is still much lower than that measured approximately in the BM (5–0.5% $O_2 \cong$ 46–4.6 μM O_2; Chow *et al.*, 2001; Harrison *et al.*, 2002; Parmar *et al.*, 2007), indicating that the ambient O_2 tension experienced *in vivo* by HSCs is not *per se* limiting for OXPHOS (Schroedl *et al.*, 2002). Other factors must, therefore, contribute to the regulation of the activity of cytochrome *c* oxidase and, as a consequence, OXPHOS.

In a study by our group, the mitochondrial respiratory activity of human $CD34^+$-HSPCs was measured for the first time, resulting in a cyanide-sensitive endogenous O_2 consumption rate under resting conditions of about 125 pmol min^{-1} per 10^6 cells (Piccoli *et al.*, 2005). This activity was depressed by oligomycin (inhibitor of the FoF1 ATP-synthase) and restored to values slightly higher than the resting respiration in the presence of the uncoupler FCCP. These results indicate that, although the respiratory activity of HSPCs is relatively low, their mitochondria are fully coupled to ATP synthesis and performing at almost the maximal respiratory capacity under resting conditions.

Staining of HSPCs with vital mitochondrion-tropic dyes reveals a heterogeneous pattern in the number and morphology of mitochondria. By double staining of human HSPCs for mitochondria and CD34, we were able to show that cells exhibiting the highest expression of CD34 displayed the lowest mitochondrial content (Piccoli *et al.*, 2005). In a further study, the mitochondrial content in HSPCs proved to correlate inversely with an additional marker, CD133, which is diagnostic for more primitive HSPCs and lost during the early phase of commitment (Handgretinger *et al.*, 2003; Piccoli *et al.*, 2007a). A similar correlation was recently found in HSPCs isolated from the BM of mice stained with the stemness marker Sca1 (Piro *et al.*, 2012). These results indicate that loss of primitive stemness is accompanied by a remodelling of the mitochondrial compartment. However, in another study, devoted to LT-HSCs, an increase in mitochondrial mass and mitochondrial membrane potential was found in cells with up-regulated CD34 (Mantel *et al.*, 2010). While apparently inconsistent with our findings, these discrepan-

cies may be explained by the fact that CD34, as a HSC marker, is poorly expressed in LT-HSCs. Therefore, a mitochondrial biogenesis is observed when HSCs lose their long-term re-populating ability, which is in agreement with the more general observation of a mitochondrial biogenesis during cell commitment and loss of multipotency. Consistent with this conclusion is the observed low retention of Rhodamine 123 in human HSCs with long-term self-renewal capacity within the $CD34^+CD38^-$ Lin^- population (Kim *et al.*, 1998; McKenzie *et al.*, 2007). Although Rhodamine 123 is commonly used to assess the efflux activity of plasma-membrane multi-drug transporters, it is also well established that, as a lipophilic cation, it specifically accumulates in active mitochondria. Moreover, very recently, it has been reported that cord blood-derived $CD34^+$ haematopoietic cells with low levels of mitochondrial mass are enriched in haematopoietic re-populating stem cell function (Romero-Moya *et al.*, 2013).

Theoretical models have been put forward inferring that the cell proliferation/differentiation may depend on the prevalence of a critical mitochondrial rather than a nuclear DNA ratio (von Wangenheim and Peterson, 1998). The energy needed to sustain proliferation/differentiation of quiescent stem cells requires bioenergetic adaptation. A four to five fold increase in ATP content of embryonic and adult stem cells has been found to follow their differentiation (Cho *et al.*, 2006; Lonergan *et al.*, 2006).

Using absolute real-time PCR, we estimated the mitochondrial DNA (mtDNA) and found it was 565 ± 100 copy number per HSPC (Piccoli *et al.*, 2007a). If it is assumed that the average number of mitochondria per HSPC is 50 ± 20 (obtained by counting the organelles specifically loaded with a fluorescent probe using a confocal microscopy), the number of mtDNA molecules per mitochondria would be more than 13 ($\pm$ 5). This number is relatively high when compared with that reported for typical differentiated mature cells [2–8 mtDNA per mitochondria (Robin and Wong, 1988)]. Interestingly, the expression of the factors controlling mtDNA replication [i.e. polymerase-gamma (POLG), mt-transcription factor A (MTFA)] has been found not to change significantly during the early phase of *in vitro* differentiation of HSCs (St John *et al.*, 2005; Cho *et al.*, 2006). This resembles that which occurs in the initial stages of mammalian embryogenesis, where regardless of the increase in mitochondrial mass, the total mitochondrial DNA in the 2 to 4/8 cell stage transition remains unchanged or even decreases (Pikó and Taylor, 1987; May-Panloup *et al.*, 2005).

Based on the aforementioned observations, we hypothesized that the initial mitochondriogenesis, in pre-committed HSPCs consists primarily of mitochondriokinesis (Kuroiwa *et al.*, 1994), with a re-distribution of pre-existing mtDNA among the newly formed organelles (Piccoli *et al.*, 2007a). Such a 'dilution' of mtDNA may help to protect the cells from the risk of mutagenic events occurring during mtDNA replication, which may drive clonal selection of transformed haematopoietic precursors or premature haematopoietic ageing (Ralph *et al.*, 2010; Norddahl *et al.*, 2011). Once the mtDNA 'density' per mitochondria has reached a 'normal' value, the successive mitochondriogenesis would start synchronizing with mtDNA replication.

Accordingly, both in embryonic and in adult stem cells, enhanced mitochondrial mass was observed following *in vitro* differentiation. Interestingly, the newly formed mitochondria re-distribute from a perinuclear aggregation to a cytoplasmic diffused state (Handgretinger *et al.*, 2003; Bavister, 2006; Cho *et al.*, 2006; Nesti *et al.*, 2007). The mitochondrial network is now well established as a dynamic entity resulting from the balance between mitochondrial fusion/fission, with the resulting organelle morphology seemingly dependent on the metabolic state of the cell (Westermann, 2010; Ferree and Shirihai, 2012). Moreover, the mitochondrial content of a cell is dependent on biogenetic rather than specific autophagic processes, also referred to as mitophagy (Novak, 2012). The latter process is necessary for the selective removal of damaged organelles. Elucidation of the mechanisms controlling mitochondrial dynamics is currently being determined for haematopoietic cell homeostasis. Conditional Atg7 (an autophagy-related protein)-deficient mice show loss of normal HSC function and die within weeks after this gene deletion (Mortensen *et al.*, 2011). The HSCs populating these mice display an accumulation of mitochondria and reactive oxygen species (ROS), as well as increased proliferation and DNA damage. Atg7-deficient HSCs also fail to reconstitute the haematopoietic system of lethally irradiated mice. These results suggest a low amount of intracellular mitochondria content is needed in HSCs to preserve their stemness.

The notion that a different metabolic profile distinguishes quiescent from differentiating stem cells, with a shift from glycolysis to mitochondrial OXPHOS, is supported by recent experimental results (Simsek *et al.*, 2010; Suda *et al.*, 2011; Takubo *et al.*, 2013). However, a key question to be answered is whether the bioenergetic shift is consequent to intrinsic nuclear reprogramming or is decided by extrinsic factors determining the fate of the stem cell. In the last few years, some important studies (briefly reviewed in the following) have contributed to the revelation that signal transduction pathways link the energy metabolism of the HSC to its self-renewing, proliferating and differentiating properties.

Mammalian target of rapamycin (mTOR), a serine/threonine PK has emerged as a key translational regulator of cellular metabolism in response to nutrient sensing (Wullschleger *et al.*, 2006) and has been found to control mitochondrial oxidative function through an YY1-PGC-1α transcriptional complex (Cunningham *et al.*, 2007). It has been reported that conditional deletion of TSC1, a negative controller of mTOR in HSCs, drives them from quiescence into rapid cycling, with increased mitochondrial biogenesis, respiratory activity and elevated levels of ROS (Chen *et al.*, 2008a). Importantly, this deletion dramatically reduced both *in vivo* haematopoiesis and self-renewal of HSCs. *In vivo* treatment with an ROS antagonist or rapamycin (an inhibitor of mTOR) restored HSC numbers and functions. The detrimental effect of up-regulated ROS in metabolically active HSCs may explain the association between quiescence and the 'stemness' of HSCs.

The TSC-mTOR complex is part of the PI3K/Akt/mTOR pathway, which is activated when stem cells proliferate in response to growth factors, nutrients and high O_2 (Yuan and Cantley, 2008). Constitutively active Akt signalling causes accelerated proliferation and depletion of HSCs (Kharas *et al.*, 2010), whereas in Akt1/Akt2 double knockout mice, HSCs remain in a constantly quiescent state and cannot differentiate into multipotent progenitors (Juntilla *et al.*, 2010). In

contrast, deletion of Pten, a PIP3 phosphatase, induces constitutively activated PI3K/Akt signalling, resulting in an accelerated cell cycle and eventually leads to HSC exhaustion (Yilmaz *et al.*, 2006; Zhang *et al.*, 2006). These data indicate that mTOR is a critical modulator of stem cell maintenance and that two tumour suppressors, Pten and Tsc1, serve as important negative regulators of PI3K/mTOR signalling in HSCs.

Recently, a different signalling pathway linking Akt/mTOR to HSC metabolism has been described. The tumour suppressor liver kinase B1 (Lkb1) is a serine/threonine kinase that positively regulates the AMP-activated PK (AMPK), which, in turn, senses the AMP/ATP ratio (Hsu and Sabatini, 2008). AMPK phosphorylation inhibits mTOR, leading to reduced cell growth and proliferation. Thus, Lkb1/AMPK signalling regulates cell metabolism in response to changes in ATP availability. Three groups have independently identified an essential role for Lkb1 in HSC maintenance (Gan *et al.*, 2010; Gurumurthy *et al.*, 2010; Nakada *et al.*, 2010). All three studies reported that conditional Lkb1 knockout mice show gradually impaired haematopoiesis, followed by transient increases in the number of haematopoietic progenitors and loss of reconstitution capacity after BM transplantation. Importantly, the typical Lkb1 null phenotype has dysfunctional mitochondria, a decreased mitochondrial membrane potential $\Delta\Psi_m$ and ATP, and down-regulation of the PPARγ co-activator 1α (PGC-1α). These outcomes are partly dependent on AMPK but not on mTOR or oxidative stress-related mechanisms, indicating that the metabolic regulation of HSCs is mediated by a novel non-canonical mechanism involving Lkb1, such as PGC-1α-mediated modulation of mitochondrial function. Interestingly, in one of these studies (Gurumurthy *et al.*, 2010), analysis of metabolic alterations in Lkb1-deficient haematopoietic cells resulted in significant alterations in lipid metabolism in the Lin^- populations. Diminished mitochondrial function and elevated fatty acid levels are consistent with a model in which Lkb1 serves as a rheostat that sets the appropriate balance of anabolic and catabolic activities in haematopoietic cells.

The interplay between maintenance of the HSCs and mitochondria-dependent lipid metabolism has been further defined by a recent study describing a new pathway that encompasses the promyelocytic leukaemia (*PML*) tumour suppressor gene [previously found to control the homeostasis of HSCs (Ito *et al.*, 2008)], PPARδ and fatty acid oxidation (FAO) (Ito *et al.*, 2012). The PML protein is the essential component of subnuclear structures (nuclear bodies) implicated in a wide variety of processes, including post-translational modifications and regulation of transcription (Bernardi and Pandolfi, 2007). PPARδ is a member of the PPAR nuclear receptor superfamily of transcription factors that control nutrient sensing and the transcriptional regulation of metabolic pathways, especially fatty acid transport and FAO (Michalik and Wahli, 2006). In this study, it was found that conditional deletion of *ppard* (coding for PPARδ) or pharmacological inhibition of mitochondrial FAO induces loss of HSC maintenance, whereas treatment with PPARδ agonists improved HSC maintenance. Moreover, the maintenance defect of $Pml^{-/-}$ HSCs could be restored by activation of PPARδ, leading to haematopoietic recovery after BM transplantation with $Pml^{-/-}$ HSCs. These findings suggested that the altered functions of $Pml^{-/-}$ HSCs are at least, in part, due to dysfunctional PPARδ signalling and FAO.

Perhaps the most exciting aspect of this work was the discovery of a link between the PML–PPARδ–FAO pathway and the control of HSC divisions. HSCs have a number of division options, which include asymmetric divisions where an HSC gives rise to a daughter HSC and a daughter progenitor (which results in HSC maintenance), or symmetric divisions [which can result alternatively in two daughter HSCs (HSC expansion) or two daughter progenitors (stemness loss)]. Using a binary assay to detect surface markers and assess cell division *in vitro*, it was found that deletion of *Ppard* or *Pml* as well as inhibition of FAO resulted in the symmetric commitment of HSC daughter cells, whereas PPARδ activation increased asymmetric cell division. The mechanism linking mitochondrial FAO to asymmetric self-renewal remains to be elucidated.

An interesting point raised by the apparent role of mitochondria in controlling HSC division is the modality by which the organelles segregate in the daughter cells. Indeed, it has been suggested that asymmetric (instead of casual) segregation of active versus nascent mitochondria might reduce the risk of oxidative damage in one of the two daughter cells, preserving a more pristine genome in HSC during self-renewing asymmetric division (Mantel and Broxmeyer, 2008).

A recently reported study revealed another feature involved in the regulation of mitochondrial metabolism in HSCs. Genetic depletion of *PTPMT1* (a PTEN-like mitochondrial phosphatase) in HSC inhibited haematopoiesis by blocking differentiation of these cells, whereas cycling of stem cell renewal was less affected (Yu *et al.*, 2013). Further analyses demonstrated that PTPMT1 deficiency altered mitochondrial metabolism and that phosphatidylinositol phosphate substrates of PTPMT1 directly enhanced fatty acid-induced activation of mitochondrial uncoupling protein 2.

The apparent relevance of the described pathways exemplifies the complicated networks in the regulation of metabolism in HSCs. Indeed, on the one hand, the maintenance of the quiescent state in the G0 state of HSC seems to rely on a glycolysis-based metabolism and proliferating multipotent precursors with a different level of commitment to active aerobic OXPHOS. On the other hand, the role of mitochondria in cycling HSC appears to be to control the balance between self-renewing cell division and symmetrically committed cell division.

See Figure 1 for a schematic overview of the points reviewed in this paragraph.

Redox signalling in HSPCs

ROS were once believed to be harmful by-products of dioxygen metabolism, but are now recognized as physiological messengers involved in controlling adaptive cell biology (Finkel, 2011). The term ROS encompasses many species, including superoxide ($O_2^{\bullet -}$), hydroxyl radical ($HO^{\bullet}$) and hydrogen peroxide (H_2O_2). Other reactive species containing nitrogen (RNS) include $NO^{\bullet}$ and peroxynitrite ($ONOO^-$). The intracellular level of these redox active molecules depends on the balance between the generating versus the scavenging

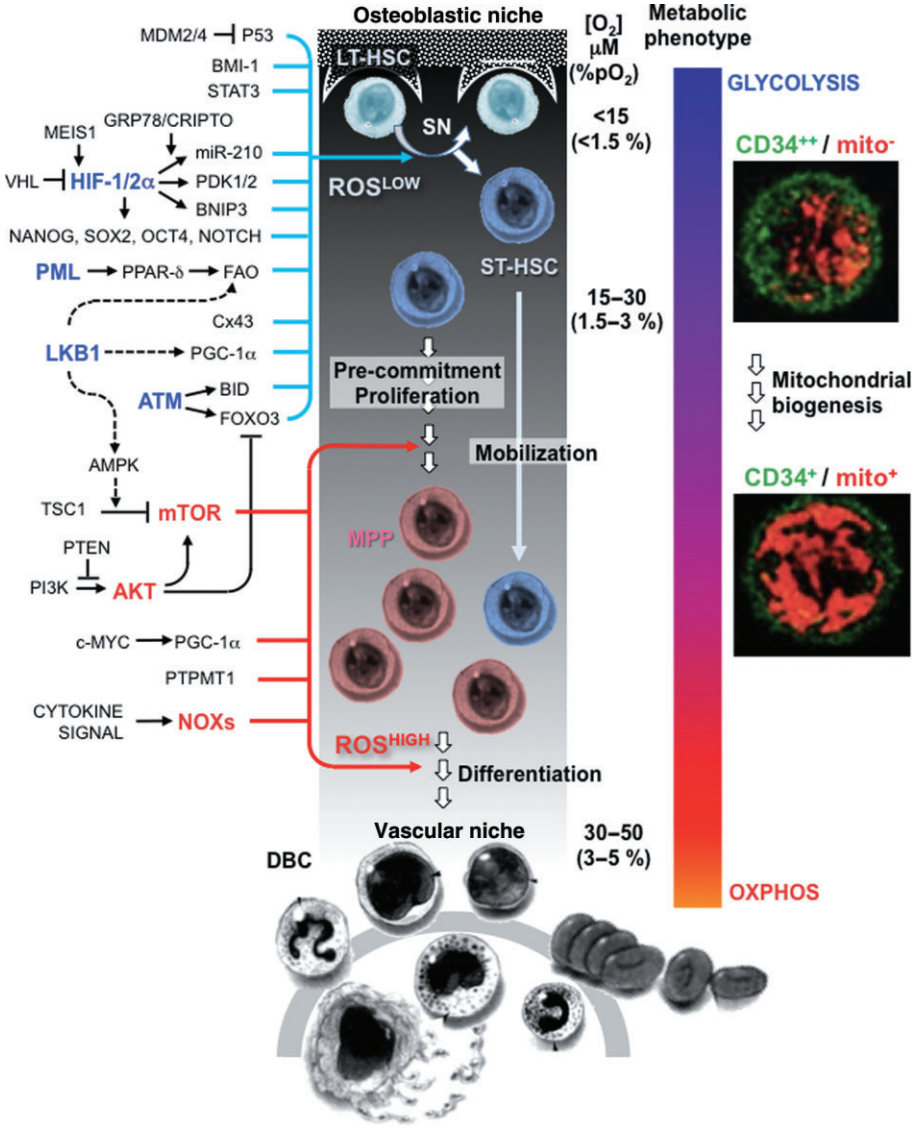

Figure 1

Redox signalling governing haematopoietic stem cell fate. Bone marrow stem cell endoblastic and vascular niches are schematically drawn on the left and bottom sides of the diagram respectively. The oxygen gradient, from the endoblastic niche to the sinusoid vessel, is also shown with the approximate values of concentration given in µM and % pO_2 units (for conversion in other units, consider that 1.0 µM O_2 = 0.77 mmHg = 5.77 kPa at 37°C). A quiescent long-term haematopoietic stem cell (LT-HSC) is elicited by niche-related factors to undergo asymmetric division. One of the two daughter cells remains in the niche, whereas the other short-term (ST)-HSC moves away from the hypoxic area and experiences a progressive increase in O_2 concentration. The ST-HSC proliferate and convert to multipotent progenitor (MPP) cells that finally are committed to one or other of the fully differentiated blood cells (DBC). The progressive loss of the stemness profile is accompanied by a metabolic shift from glycolysis to oxidative phosphorylation. The two pictures on the right-hand side are confocal images of cells co-stained for the stemness marker CD34 and the mitochondrial compartment showing an increase in mitochondrial mass in the $CD34^{low}$-HSPC. The increased number of functional mitochondria in the pre-commitment stage of the ST-HSC/MPP provides the cell with the energy-generating systems needed to cope with a proliferating/differentiating metabolism. On the left-hand side of the picture: schematic depictions of the main signalling pathways reported to control the quiescence/maintenance of the HSCs or to induce their proliferation/differentiation (the prime factors of the pathways are highlighted in blue and red respectively). All the pathways are linked directly or indirectly to the regulation of the cellular redox state with the low and high reactive oxygen species (ROS^{LOW} and ROS^{HIGH}) level priming the HSC maintenance and commitment respectively. See text for definitions of the abbreviations.

systems. According to a 'window hypothesis', a cell-type-dependent threshold level of such reactive species exists below which the altered oxido-reduction state of the cell is transduced in a physiological response.

Redox signalling has been shown to be involved in cell fate decision under the pressure of challenging conditions leading to activation of growth, proliferation, differentiation or cell cycle arrest, senescence and apoptosis (Sauer *et al.*,

2001; Dröge, 2002; Finkel, 2003). The amount and duration of ROS are likely to provide a combination of conditions that result in apparently contradictory cell responses. So, a short-lived ROS production of low intensity is transduced in a signal leading to cell proliferation or differentiation, whereas the continuous generation of relatively large amounts of ROS elicits growth arrest and eventually cell death (Boonstra and Post, 2004; Burhans and Heintz, 2009; Chiu and Dawes, 2012). The specific chemical species involved and/or their cellular compartmentalization are also important elements in determining the signalling outcome.

ROS such as $O_2^{\bullet-}$ and H_2O_2 are generated from a number of sources including mitochondrial RC complexes, NADPH oxidases (NOXs), xanthine oxidase, cytochrome P450 and uncoupled NOS. Importantly, ROS generation is considered for some of these sources as an unwanted by-product of oxygen metabolism, as in the case of the mitochondrial RC, whereas for others, it is the intended product of the enzymatic reaction, as in the case of the NOXs. This distinction, however, may require more in-depth re-consideration. The superoxide anion $O_2^{\bullet-}$ represents the seminal ROS product wherefrom all the others derive. As $O_2^{\bullet-}$ is generated by a bimolecular reaction, it is generally assumed that the oxygen concentration has a significant effect on the total amount of ROS. However, this conviction is challenged by experimental evidence and needs further consideration. The $O_2^{\bullet-}$ reacts with NO to generate $ONOO^-$, whereas it can be quickly converted to H_2O_2 by superoxide dismutases (SODs) such as Cu/Zn SOD (SOD1), MnSOD (SOD2) or extracellular SOD (SOD3). H_2O_2 is catalysed by catalase, glutathione peroxidases and the thioredoxin–peroxiredoxin system to water. Because H_2O_2 is relatively stable, membrane permeant and does not react with NO, it has been proposed to represent the main second messenger in physiological redox signalling.

Identifying the direct molecular target(s) of ROS in each cell type is important in understanding the cellular mechanism of redox regulation and is still a contentious issue. Signalling by ROS and RNS occurs through chemical reactions with specific atoms of target proteins; thus, recognition is at the sub-molecular, atomic level, and consequently has the potential for recognition of many ROS/RNS-specific receptors (Nathan, 2003). Nevertheless, with most of the redox-regulated proteins studied, the targeting of specific reactive groups within specific targeted proteins is evident. While in many of them, reactive cysteine residues function as redox sensors, others use a redox-sensitive metal iron (Baldwin and Benz, 2002; Ilbert *et al.*, 2006).

The thiol-based 'peroxide receptors' or 'sensors' that react with H_2O_2 form a hierarchy in the oxidation of thiols in other proteins by interacting with them (Toledano *et al.*, 2004). The model proposes that oxidation of most target proteins is not conducted directly, but rather mediated through the oxidation of a subset of very reactive thiols in sensor proteins, which, in turn, facilitate the oxidation of other target proteins through selective protein–protein interactions (Winterbourn and Hampton, 2008; Finkel, 2011). A redox signal is transmitted from the site of ROS generation through the sensor protein to its final destination, which may reside, as in the case of the transcription and replication factors, in the nucleus. Thus, different redox states within different subcellular compartments may present an additional constraint on the transmission of a redox signal.

In the past decade, specific interest and intense investigation have been addressed to the role of the redox status in controlling the balance between quiescence, self-renewal and differentiation of stem cells including HSCs and their function (Eliasson and Jönsson, 2010; Sardina *et al.*, 2012; Urao and Ushio-Fukai, 2013). When isolated HSCs are stained with the ROS-sensitive fluorescent probe dichlorofluorescein (DCF), two subpopulations can be distinguished based on their basal ROS level, DCF^{high} and DCF^{low}. Isolation and further characterization by serial transplantation of the two fractions reveals that the DCF^{low} HSC population retains a higher reconstitution ability (i.e. higher self-renewal potential) than the DCF^{high} population (Jang and Sharkis, 2007). Interestingly, the DCF^{high} fraction had higher myeloid differentiation capacity than the DCF^{low} cells, indicating that the DCF^{high} fraction is 'myeloid shifted,' a characteristic of senescent HSCs. Treatment of the DCF^{high} fraction with antioxidants, p38 MAPK or mTor inhibitors restored their colony-forming capacity *in vitro*. The authors proposed that the DCF^{low} fraction represents the low cycling quiescent HSC subset located in the hypoxic endoblastic niche. In a study on Drosophila haematopoietic progenitors, using genetic tools, it was shown that scavenging or moderately increasing ROS levels decreases or stimulates HSPC differentiation respectively (Owusu-Ansah and Banerjee, 2009). Thus, the effect of ROS on HSC function probably depends on its concentration and the exposure period.

Consistent with the importance of redox signalling in regulating division and driving differentiation of HSPC is the observation that a number of haematopoietic growth factors or cytokines stimulate signalling events, leading to cell growth (Sattler *et al.*, 1999; Iiyama *et al.*, 2006) or promote HSC mobilization into the circulation through the formation of H_2O_2 (Tesio *et al.*, 2011). ROS-dependent HSC proliferation plays an important role in the early steps of haematopoietic reconstitution after HSC transplantation (Lewandowski *et al.*, 2010).

The mechanism controlling the HSC redox status has been investigated using relevant gene-targeted mice. Conditional deletion of these genes in HSCs results in alterations in the cellular ROS balance towards a pro-oxidative state. This induces the HSCs to egress from quiescence, to lose self-renewal capacity, to accelerate proliferation/differentiation, to promote migration and to induce genome instability, thereby causing rapid depletion of the haematopoietic reserve (reviewed in Ushio-Fukai and Urao, 2009; Eliasson and Jönsson, 2010; Sardina *et al.*, 2012; Urao and Ushio-Fukai, 2013). Importantly, treatment with an antioxidant in most cases restores the altered HSPC phenotype.

These studies have helped to identify the intertwined ROS-related regulatory network in HSCs comprising: the transcription factor FOXOs (Miyamoto *et al.*, 2007; Tothova *et al.*, 2007); p38 MAPK (Ito *et al.*, 2006; Miyamoto *et al.*, 2007); PI3K–Akt1/2 signalling (Juntilla *et al.*, 2010; Kharas and Gritsman, 2010), PTEN (Yilmaz *et al.*, 2006), TSC1/mTOR (Chen *et al.*, 2008b), ATM-CDK-inhibitors $p16^{Ink4a}$ and $p19^{Arf}$ (Ito *et al.*, 2004; 2006), ATM-BID (Maryanovich *et al.*, 2012), the polycomb protein Bmi-1 (Lessard and Sauvageau, 2003; Park *et al.*, 2003; Oguro *et al.*, 2006; Liu *et al.*, 2009), P53-

Mdm2 (Abbas *et al.*, 2010; 2011), the nuclear transcription factor Stat3 (Hankey, 2009; Mantel *et al.*, 2012) and connexin-43, (Taniguchi Ishikawa *et al.*, 2012). Of note, many of the transduction pathways that have been identified interact with each other, contributing by feed-forward and feedback mechanisms to fine-tune the redox homeostasis of HSPCs. Nevertheless, in some instances, it is unclear which ROS generating system is involved following targeted gene depletion. Although there are several sources of ROS, NADPH oxidases (NOXs) and mitochondria are the major ROS-generating systems in BM-derived HSPCs.

NADPH oxidase is a cell membrane bound di-haeme-containing enzyme that catalyses the transfer of electrons (one at a time) from NADPH to O_2, leading to the formation of the superoxide radical anion $O_2^{\bullet-}$ (Cross and Segal, 2004). No other activity has been reported for NOX; thus, it is the only enzyme in the cell that 'deliberately' generates ROS. The best-characterized isoform NOX2 participates in the host-defence function in macrophagic blood cells by provoking the bactericidal 'oxidative burst'. This reaction is tightly controlled and the catalytic oxidase subunit, which is usually silent, is promptly activated (within seconds) by the recruitment of a number of cytosolic regulatory subunits. This event is triggered by a signalling cascade, which starts from external stimuli and leads to the activation of the GTP-binding protein Rac1/2 and to phosphorylation of p47, both regulatory NOX2 subunits (Cross and Segal, 2004). In the last few years, NOX2 along with other evolutionary-related isoforms (NOX1, NOX3, NOX4, NOX5, DUOX1 and DUOX2) have been discovered in many other cell types not involved in host defence mechanisms (Cheng *et al.*, 2001; Bokoch and knaus, 2003) and are thought to be involved in redox signalling (Sauer *et al.*, 2000; 2001; Dröge, 2002; Bokoch and knaus, 2003; Finkel, 2003). These additional NOX2 isoforms rely on different subunit assembly patterns and appear to be differently regulated (Lambeth *et al.*, 2007; Petry *et al.*, 2010).

By utilizing G-CSF-mobilized human $CD34^+$-HSPCs, we were the first to provide functional evidence of O_2 consumption attributable to NOX activity [i.e. ≅80 pmol min^{-1} per 10^6 cells (Piccoli *et al.*, 2005)]. This was further confirmed at the transcriptional and translational level, revealing the presence of multiple isoforms of NADPH oxidase (i.e. NOX1, NOX2 and NOX4) (Piccoli *et al.*, 2007b). Moreover, in a sequential study using high-resolution imaging of HSPC membranes, we immunodetected the presence of NOX in membrane 'raft'-like microcompartments where the assembly/activation of the NOX components may be functionally integrated to create redox signalling platforms (Frassanito *et al.*, 2008). The NOX activity in $CD34^+$-HSPCs is constitutive and only slightly stimulated by PMA or G-CSF (Piccoli *et al.*, 2005; 2007b). $O_2^{\bullet-}$ generated outside the cell can dismutate to form the freely diffusible H_2O_2. Consistently, we found that the extracellular isoform SOD3 was expressed in HSPCs (Piccoli *et al.*, 2007b). H_2O_2 can enter into the cell and alter its redox status. In fact, we detected intracellular ROS by using the DCF probe and found that its level was largely (but not completely) decreased by specific inhibitors of NOX or by pre-treatment of $CD34^+$-HSPCs with externally added catalase or an inhibitor of the PI3K/Akt pathway (Piccoli *et al.*, 2007b).

The catalytic properties of NOXs make them suited to function as oxygen sensors in the HSPC physiological environment. Indeed, the reported K_M for O_2 of NOXs is about 10–15 μM (Gabig and Babior, 1979; Chen *et al.*, 2005), implying that their steep activity-slope falls in the range of the O_2 concentration estimated in the BM from the endosteum to the sinusoidal vasculature (i.e. from <5 to 30–40 μM respectively). On this basis, we have proposed a model whereby the NOX4 works as a primary oxygen sensor in HSPCs (Piccoli *et al.*, 2007b). NOX4 is, apparently, the only NOX isoform that does not require regulatory subunits and is constitutively active (Lambeth *et al.*, 2007). Thus, the amount of NOX4-generated $O_2^{\bullet-}$ reflects the range of the environmental O_2 experienced by the HSPCs. Furthermore, we proposed that intracellular H_2O_2 induces the activation of the other NOX isoforms by stimulation of their regulatory subunits. Indeed, most of the upstream factors, which activate NOX1/2, are themselves targets of redox control (Pendyala and Natarajan, 2010), thus placing NOX1/2 downstream and upstream of redox signalling. This, in turn, provides a positive feedback regulatory loop that gradually enhances intracellular ROS signalling upon displacement of the HSPCs from a hypoxic endoblastic function to a vasculature function activating/fostering therein proliferation/differentiation by HSPC reprogramming.

A number of reported findings, summarized in the following, support this model: (i) hypoxic conditions (<5% pO_2) is a well-established setting for both quiescence maintenance and induction of HSPC from cord blood and BM in *ex vivo* cultures (Cipolleschi *et al.*, 1993; Danet *et al.*, 2003; Ivanovic *et al.*, 2004; Hermitte *et al.*, 2006; Shima *et al.*, 2010); (ii) inhibitors of NOX preserve the progenitor features of HSPCs under normoxic conditions (Fan *et al.*, 2007); (iii) activated Ras promoted both survival and growth factor-independent proliferation of $CD34^+$-HSPCs through stimulation of NOX activity and ROS generation leading to up-regulation of the expression of D cyclins (Hole *et al.*, 2010); (iv) NOX-related ROS are generated by haematopoietic cytokines and play key roles in the activation of receptor-mediated signalling and in the cell cycle progression (Iiyama *et al.*, 2006; Woolley *et al.*, 2012); and (v) ROS derived from NADPH oxidase play an important role in redox signalling linked to BM stem/progenitor cell post-ischaemic mobilization, homing and differentiation, thereby promoting neovascularization in hypoxic areas (Ushio-Fukai and Urao, 2009; Urao *et al.*, 2012).

Moreover, NOX-mediated ROS signalling has been reported in the physiology of other stem cell populations, implying a more general role of NOXs in controlling stem cell biology. NOX-mediated ROS production promotes differentiation of (i) BM mesenchymal stem cells towards osteoclasts (Lee *et al.*, 2005), neurons (Wang *et al.*, 2007), adipocytes (Kanda *et al.*, 2011); mouse embryonic stem (ES) cells towards endothelial cells (Sauer *et al.*, 2005; Schmelter *et al.*, 2006), smooth muscle cells (Xiao *et al.*, 2009), phagocytic cells (Hannig *et al.*, 2010) and cardiomyocytes (Sauer *et al.*, 2000; Li *et al.*, 2006; Buggisch *et al.*, 2007). The ROS-mediated signalling appears to be time-dependent, being activated during the early stages of ES cell differentiation and then down-regulated during later stages. During the differentiation process, antioxidative genes are down-regulated (Saretzki *et al.*, 2004), while NOXs 1/2/4 are up-regulated (Buggisch *et al.*, 2007).

ROS are also produced from mitochondria as a consequence of aerobic metabolism (Murphy, 2009; Dröse and Brandt, 2012; Papa *et al.*, 2012). The electron transport in mitochondrial RC starts with the extraction of electrons from NADH (complex I) or $FADH_2$ (complex II) generated in the tricarboxylic acid cycle. Electrons are then transferred via ubiquinone to complex III and finally to complex IV. Electron leak during respiration leads to the formation of $O_2^{\bullet-}$ due to the incomplete reduction of O_2 at complex I and complex III (Dröse and Brandt, 2012). $O_2^{\bullet-}$ formed at complex I is released into the mitochondrial matrix, whereas $O_2^{\bullet-}$ formed at complex III can be released either to the matrix or to the intermembrane space. Recent evidence that complex II may also be a site of ROS generation has been provided. Inhibition of complex III or IV and/or establishment of a large electrochemical gradient are all conditions that enhance electron leak by a mechanism known as 'reverse electron flow'. Conversion of $O_2^{\bullet-}$ in H_2O_2 by outer mitochondrial membrane-associated or intra-mitochondrial SOD1 and SOD2, respectively, generates a relatively long-lasting membrane-permeant reactive species that, when escaped from the armoury of other antioxidant enzymes, can flux from the mitochondria to the cytoplasm. The role of mitochondria-related ROS-mediated signalling in the adaptive response to physiological stressors/inducers is recognized in a number of cell systems, including HSPCs as mentioned before (Hamanaka and Chandel, 2010; Rehman, 2010; Mantel *et al.*, 2011; Rigoulet *et al.*, 2011; Finkel, 2012; Lenaz, 2012; Maryanovich and Gross, 2012; Papa *et al.*, 2012).

An intriguing aspect reported in various cell systems is the cross-talk between NOX and mitochondrial ROS (Daiber, 2010; Dikalov, 2011). It has been suggested that mitochondrial H_2O_2 regulates NOX activity. Conversely, NOX activation induces mitochondrial H_2O_2 formation by a number of different mechanisms. Given that H_2O_2 is a highly diffusible molecule, cross-talk between NOXs and mitochondrial ROS may represent a positive feed-forward mechanism that promotes sustained H_2O_2 production and activation of redox signalling. This may be part of a more general phenomenon described as 'ROS-induced ROS release' (Zorov *et al.*, 2006; Zinkevich and Gutterman, 2011). Whether this regulatory mechanism is involved in stem and progenitor cell function is the subject of future investigation (Mantel *et al.*, 2011). Considering the stability and membrane-permeant properties of H_2O_2, redox signalling in HSPCs can also be affected by H_2O_2 production from surrounding cells.

See Figure 1 for a schematic overview of the points reviewed in this paragraph.

HIF-1-mediated signalling in HSPC: beyond O_2-sensing and adaptation to hypoxia

While a moderate increase in the ROS level has been shown to trigger a proliferative response and probably to provide conditions making HSPC more responsive to differentiation stimuli, in a low ROS level state, less active cells with preserved stemness properties have been identified (Jang and Sharkis, 2007). In this regard, it is not clear whether the 'low ROS level' regime is by itself functional to the maintenance of the undifferentiated state through a signal transduction pathway different from that attained under 'high ROS level' conditions.

The low ROS level displayed by the subset of more primitive HSPCs is thought to be a direct consequence of the hypoxic features of the endoblastic niche where this HSPC population resides (Chow *et al.*, 2001; Parmar *et al.*, 2007). This low-oxygen environment is not only tolerated by HSCs, but also appears to be essential for their function (Bradley *et al.*, 1978; Katahira and Mizoguchi, 1987; Koller *et al.*, 1992; Cipolleschi *et al.*, 1993; LaIuppa *et al.*, 1998; Danet *et al.*, 2003; Kubota *et al.*, 2008; Lo Celso *et al.*, 2009; Eliasson and Jönsson, 2010).

The main experimental data supporting the localization of HSCs in a hypoxic BM niche are as follows: (i) administration of a perfusion tracer into mice showed that HSCs were relatively enriched in a low-perfusion cellular compartment in the BM (Parmar *et al.*, 2007) and retained pimonidazole, a probe that selectively forms thiol-adducts under hypoxic conditions; (ii) administration of tirapazamine, a toxin selective for hypoxic cells, promoted loss of HSCs *in vivo*; and (iii) LT-HSCs proved to be pimonidazole$^+$ *in vivo* (Simsek *et al.*, 2010; Takubo *et al.*, 2010).

Taken together, these findings support a model in which the hypoxic character of LT-HSCs is determined by their position within the BM. However, in contrast to the simple O_2 gradient model for the BM hypoxic niche, two-dimensional observations of a segment of the BM suggest that a large fraction of the LT-HSCs reside adjacent to the vasculature (Kiel *et al.*, 2005; Sugiyama *et al.*, 2006). This would imply the existence of a different location for the HSCs defined as vascular, which, even considering the poor perfusion through the endosteal capillaries, is far from being a severe hypoxic environment. Thus, it is possible that subpopulations of HSCs exist that reside in different specific locations, in different cell cycle and/or metabolic states and that HSCs dynamically move within the BM acquiring distinct environment-dependent phenotypes (Lo Celso *et al.*, 2009; Xie *et al.*, 2009). The hypoxic endosteal niche would house quiescent non-cycling HSCs.

A key component for cellular and systemic responses to reduced O_2 availability is the transcription factor hypoxia inducible factor-1 (HIF-1) (Semenza, 2009). HIF-1 is a heterodimeric protein consisting of an O_2-regulated α subunit and a constitutively expressed β subunit (Wang and Semenza, 1995). Under normoxic conditions, HIF-1α is hydroxylated at two specific proline residues in the O_2-dependent degradation domain (Kaelin and Ratcliffe, 2008) and at a specific asparagine residue in the C-terminal transactivation domain by three prolyl hydroxylases, PHD1–3 and the asparagine hydroxylase FIH-1, respectively; all require O_2, Fe^{2+}, 2-oxoglutarate and ascorbate for enzymatic activity (Epstein *et al.*, 2001). Prolyl-hydroxylated HIF-1α is recognized by the von Hippel-Lindau (VHL) tumour suppressor protein, which recruits an ubiquitin-ligase complex, leading to ubiquitination and subsequent proteasomal degradation. Under hypoxic conditions, HIF-1α prolyl hydroxylation is suppressed, stabilizing the HIF-1α protein, which moves into the nucleus where it interacts with its cognate partner, HIF-1β

(Kaelin and Ratcliffe, 2008). HIF-1α : HIF-1β heterodimers bind to hypoxia response elements (HREs) in numerous target genes and activate transcription. HIF-2α is an important isoform of HIF-1α, which displays a low level of redundancy. The two isoforms are believed to play a differential role under conditions of acute or chronic hypoxia (Koh and Powis, 2012).

Thus, HIF activity is regulated by an exceptionally tight, two-level mechanism in which HIF-1/2α stability is regulated through O_2-dependent proline hydroxylation by PHDs (PHD-VHL-proteasome) and its transcriptional activity through the action of O_2-dependent asparagine hydroxylation by FIH1. It has been reported that HIF hydroxylases are present in the cell in limited amounts and that the intracellular physiological concentration of O_2 is below the apparent K_M in this reaction (estimated to be around 230 and 90 μM for PHDs and FIH1 respectively (Hirsilä *et al.*, 2003; Koivunen *et al.*, 2004). Thus, hydroxylases have the capacity to respond to significant physiological changes in intracellular oxygen concentrations, linking them to the regulation of HIF. However, considering that the estimated range of O_2 concentration from the sinusoidal vessels to the endoblastic niche is from 40–50 to 5 μM, the HIF hydroxylases are likely to work in a low-activity range with respect to their catalytic potential. Indeed, the activation of HIF is also controlled by a number of O_2-independent mechanisms at the post-transcriptional and post-translational level (Koh *et al.*, 2008). As discussed below, oxygen sensing by the hydroxylases may also proceed via an indirect process mediated through the generation of ROS in the mitochondria under hypoxic conditions.

HIF-1 activity promotes a switch from oxidative metabolism to glycolysis (reviewed in Semenza, 2011) through the transcriptional activation of genes encoding glucose transporters such as glucose transporter 1, glycolytic enzymes such as LDHA, and metabolic regulatory enzymes such as pyruvate dehydrogenase kinase 1 (PDK1) (Iyer *et al.*, 1998; Suda *et al.*, 2011). Importantly, HIF-1α positively controls the expression of the isoform cytochrome *c* oxidase 4-2, a subunit of the cytochrome *c* oxidase and of a mitochondrial protease Lon, which degrades the 'normoxic' cytochrome *c* oxidase 4-1(Fukuda *et al.*, 2007). This change in the subunit composition of the cytochrome *c* oxidase optimizes the efficiency of respiration under hypoxia and reduces ROS production. Moreover, HIF-1 induces BNIP3, which triggers mitochondrial-selective autophagy (Zhang *et al.*, 2008; Bellot *et al.*, 2009) and the microRNA-210, which blocks the assembly of Fe/S clusters that are required for OXPHOS (Chan *et al.*, 2009).

HIF-1 mRNA and protein are highly expressed in LT-HSCs (Simsek *et al.*, 2010; Takubo *et al.*, 2010). Conditionally HIF-1α-deleted HSCs lose their capacity for marrow reconstitution during serial BM transplantation, enter the cell cycle from G0 in a p16Ink4a/p19Arf-dependent manner, proliferate, and display reduced tolerance to stressors or aging (Takubo *et al.*, 2010). Overstabilization of HIF-1α by biallelic loss of VHL induces cell cycle quiescence in HSCs and their progenitors but results in impaired transplantation capacity. In contrast, monoallelic loss of VHL induces cell cycle quiescence and improved BM engraftment during BM transplantation. These results indicate that there is an optimal HIF-1α protein level for HSC maintenance with an aberrant activation of its stabilization being detrimental to the HSC biology. Accordingly, long-term *in vitro* treatment of LT-HSCs with a PHD inhibitor, dimethyloxalylglycine, which also stabilizes HIF-1α, attenuates stem cell capacity during BM transplantation (Eliasson *et al.*, 2010). In a recent study it was demonstrated that regulation of glycolysis by PDK functions as a metabolic checkpoint for cell cycle quiescence in HSCs, further supporting the role of a HIF-1 in conditioning the fate of HSC through metabolic control (Takubo *et al.*, 2013). PDK overexpression in glycolysis-defective HSCs restores glycolysis, cell cycle quiescence and stem cell capacity, while loss of both Pdk2 and Pdk4 attenuates HSC quiescence, glycolysis and transplantation capacity. Moreover, treatment of HSCs with a PDK mimetic promotes their survival and transplantation capacity.

However, the glycolytic phenotype of LT-HSCs appears to be dependent on transcriptional activation of HIF-1α through the HSC-specific transcription factor MEIS1 (a homeobox protein) and not merely a product of a hypoxic condition (Simsek *et al.*, 2010).

HIFs also interact with core stemness networks because HIF-2α can up-regulate OCT4 expression and modulate Wnt/β-catenin signalling, while silencing of HIF-2α and HIF-3α decreases the expression of transcription factors, NANOG, SOX2 and OCT4 (Covello *et al.*, 2006; Forristal *et al.*, 2010; Mazumdar *et al.*, 2010). One of the heat shock proteins, GRP78, and its ligand, Cripto, regulate HSC quiescence and maintain HSCs in hypoxia as an intermediary of HIF-1α (Miharada *et al.*, 2011). The Cripto promoter region has an HRE, onto which the stabilized HIF-1 complex binds. Moreover, it has been found that hypoxia synergizes with Notch to inhibit differentiation of myogenic and neural precursor cells. This effect requires the interaction between the transcriptionally active form of HIF-1α and the intracellular domain of Notch (Gustafsson *et al.*, 2005).

In addition to the low oxygen availability, in normoxic conditions HIF-1α is also responsive to a variety of stimuli, including various growth factors, cytokines and hormones. It has been found that many of these non-hypoxic stimuli use ROS in the regulation of HIF-1α (Kietzmann and Görlach, 2005; Pouysségur and Mechta-Grigoriou, 2006). The notion of redox control/modulation of HIF-1α activity has been supported, in the last two decades, by a large body of evidence, although the sources and mechanisms of ROS generation are still controversial. H_2O_2 and NO donors were found to stabilize HIF, and genetic and pharmacological interventions that affect ROS generation have been shown to affect the accumulation of HIF-1α (Brüne and Zhou, 2003; Kietzmann and Görlach, 2005). In various studies, NOX1-2 isoforms have been suggested as important sources of ROS in the regulation of both HIF-1 and -2α (Görlach *et al.*, 2001; Kim *et al.*, 2002; Goyal *et al.*, 2004; Maranchie and Zhan, 2005; Bonello *et al.*, 2007).

Our group provided evidence that G-CSF-mobilized $CD34^+$- and $CD133^+$-HSPCs stabilized a cytoplasmic form of HIF-1α under normoxic conditions (Piccoli *et al.*, 2007c). It was shown that HIF-1α stabilization correlated with the down-regulation of pVHL and was positively controlled by NADPH-oxidase-dependent production of ROS. Conversely, normoxic stabilization of HSPCs was not observed in BM-resident HSPCs, thus indicating a specific O_2-

independent post-transcriptional control of HIF in mobilized HSPCs. Normoxic stabilization of the HIF-2α was also recently assessed in the same cell phenotype (unpublished data). This finding led us to propose that HIF functions as a mediator of HSPCs recruitment to injured ischaemic tissues while preserving the maintenance of the undifferentiated state. Accordingly, hypoxic tissues, like cardiac peri-ischaemic areas, promote HSC mobilization by HIF-1-mediated release of stromal-derived factor 1, which acts at the level of BM, recruiting undifferentiated HSCs into the circulating blood (Ceradini *et al.*, 2004). Following a chemotactic gradient, HSCs can reach the hypoxic tissue and provide it with a source of endothelial precursors that could help in the re-vascularization process, as recently suggested by a study showing that endothelial cells are an intrinsic component of adult myeloid lineage differentiation (Bailey *et al.*, 2006). Given the interchangeable features shared by chemokines and growth factors (Vandervelde *et al.*, 2005), it is possible that G-CSF conditioning might mimic the physiological BM-mobilization of HSCs (Powell *et al.*, 2005) under crisis conditions. This mechanism in circulating HSPCs might also support the re-population of exhausted BM niches in physiological conditions, as well as in clinical practice during BM reconstitution/transplantation.

The main mechanism suggested for the ROS-mediated activation of HIF is the redox modification of the catalytic site of PHD1-3 and FIH1. As described earlier, both these types of hydroxylases are dioxygenases that require Fe^{2+} and are affected by changes in the levels of this co-factor. Fe^{2+}, which is oxidized during an occasional decoupled pathway in the hydroxylation reaction, requires ascorbate to be regenerated. Hence, the accumulation of ROS would inactivate PHDs and FIH1 and, consequently, stabilize HIF-1 as a consequence of direct oxidation of Fe^{2+} or depletion of antioxidants (Gerald *et al.*, 2004; Pouysségur and Mechta-Grigoriou, 2006).

A subject of intense investigation and debate is the role of ROS-mediated signalling as an important, if not essential, factor in controlling the HIF-1 activity even under conditions of limited O_2 availability. Although counterintuitive, a large body of accumulated evidence shows that under hypoxia, cells generate ROS at levels that, depending on the conditions, can be even higher than that observed under normoxia (Chandel *et al.*, 1998). Using genetically manipulated cells lacking either SOD1/2 or GPX1/catalase, it was found that hypoxic stabilization of HIF-1α occurs specifically with the latter, thus suggesting that H_2O_2 is the ROS likely to be involved in this process (Brunelle *et al.*, 2005).

A series of studies supports a role for mitochondrial RC in the generation of the ROS involved in the hypoxic response. It was shown that HIF-1α activation was impeded in ρ^0 cells, which lack functional mitochondrial DNA, and in cells treated with inhibitors of the mitochondrial RC complexes. Further in-depth studies demonstrated that in the electron transport chain, complex III is an important source of hypoxic ROS (Brunelle *et al.*, 2005; Guzy *et al.*, 2005). Pharmacological and genetic evidence suggests that the ubiquinone (Q) cycle of complex III is the main site of ROS generation linked to hypoxic stabilization of the HIF-1α protein (Chandel *et al.*, 1998; 2000; Brunelle *et al.*, 2005; Guzy *et al.*, 2005; Mansfield *et al.*, 2005). Complex III can release O_2 into the mitochondrial intermembrane space and subsequently into the cytosol (Muller *et al.*, 2004). In accord with these observations, it was found, using cytochrome *b* mutant cybrids, that when hypoxic ROS production was up-regulated, HIF-1α was stabilized. Moreover, the mitochondrial-targeted antioxidant Mito-Q inhibited the ROS-mediated activation of the HIF pathway in hypoxia (Bell *et al.*, 2007). On the other hand, several other reports have challenged these data, demonstrating that in ρ^0 cell lines, HIF-1α was stabilized under severe hypoxic conditions (Doege *et al.*, 2005).

To note, under severe hypoxia (anoxia), ROS production is impaired by the absence of O_2 but nevertheless HIF-1 is activated (Schroedl *et al.*, 2002). Importantly, cells that reside under hypoxia do not undergo cell death, whereas a sustained period of anoxia causes cell death. Thus, reduction of O_2 depletion under hypoxia might be a way to prevent the creation of harmful anoxic conditions. As the RC is the primary consumer of intracellular O_2, the HIF-mediated inhibition of the mitochondrial metabolism prevents the depletion of O_2, maintaining it at acceptable levels of hypoxia. The failure to stabilize HIF-1α under hypoxia in cells with impaired mitochondrial function was interpreted as being the result of reduced mitochondrial respiration and intracellular O_2 redistribution. According to this proposal, known as the oxygen redistribution model, this led to an increase in O_2 availability and, consequently, the relief of hypoxia, rather than reduced ROS production by the electron transfer chain (Hagen *et al.*, 2003). However, the two proposed mechanistic models are not necessarily reciprocally excluding and can both contribute an explanation of the role of functional mitochondria in the hypoxic induction of HIF.

See Figures 1 and 2 for a schematic overview of the points reviewed in this paragraph.

O_2-binding/releasing globins: a new entry in HSPC physiology

A further adaptive response to low O_2 tension found in different cell types is the induced expression of O_2-binding/releasing globins comprising myoglobin (Mb) and Hb and the recently discovered cytoglobin (Cygb) and neuroglobin (Ngb). We have obtained functional and molecular evidence that human HSPCs express Mb at a level comparable with that of a muscle-derived cell line. A search for other globins revealed the significant expression of Ngb but not of Cygb. Moreover, confocal microscopy immune-detection strikingly demonstrated nuclear localization of Mb in cell subsets expressing a high level of CD34, whereas Ngb was homogeneously distributed in the cytoplasm of all the HSPCs (unpublished data).

The dogma that Mb is exclusively present in cardiac or skeletal muscle of vertebrates has been recently challenged (Fraser *et al.*, 2006; Roesner *et al.*, 2008) and, notably, Mb expression has been found in a number of non-myogenic human tumours as well as in cancer-derived cell lines (Flonta *et al.*, 2009; Kristiansen *et al.*, 2010; Gorr *et al.*, 2011; Oleksiewicz *et al.*, 2011). Ngb is almost exclusively expressed in the nervous system and in the retina (Burmester *et al.*, 2000; Reuss *et al.*, 2002; Schmidt *et al.*, 2003). However, in

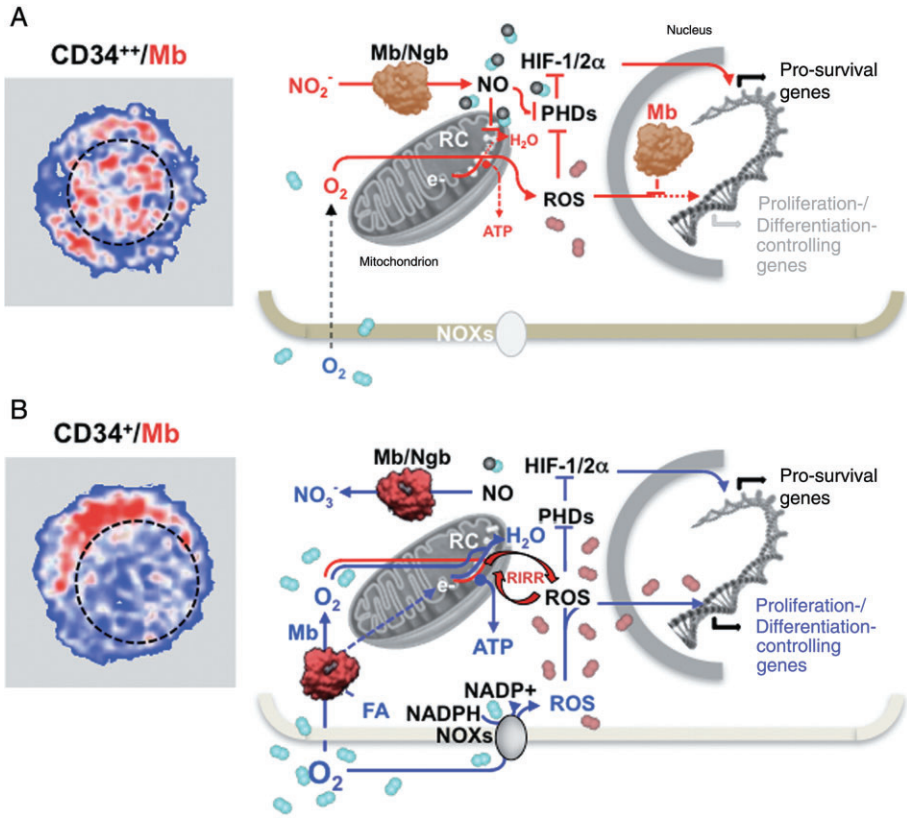

Figure 2

Schematic view of the suggested functional role of globins under hypoxic and normoxic conditions in quiescent and early committed HSPCs. The pictures on the left are artificial colour images of $CD34^{++}$ (A) and $CD34^{+}$ (B) in human HSPCs immunostained with a myoglobin (Mb)-Ab. The Mb-related fluorescence signal is shown in red and the nuclear compartment outlined with a dashed black line. The schemes on the right illustrate the suggested functions of Mb and neuroglobin (Ngb) in the context of hypoxic (A) and normoxic (B) conditions in early and late HSPCs respectively. The prevailing de-oxygenated and oxygenated state of the globins is highlighted with a different colour tone in (A) and (B). It is shown that under hypoxia (A) the de-oxygenated form of Mb/Ngb function as a nitrite reductase generating NO. This inhibits the mitochondrial respiratory chain (RC) and the oxidative phosphorylation promoting electron leak to O_2 with formation of ROS, which, along with NO, inhibit the prolyl-hydroxylases (PHDs) thereby stabilizing the hypoxia inducible factors (HIF-1/2α). The transcriptional activity of HIF induces the expression of a set of pro-survival genes. The nuclear localization of Mb under this condition prevents ROS from scavenging the redox-linked activation of NFs/genes involved in the proliferation/differentiation of HSPCs or oxidative damage of DNA. Under normoxic conditions (B) the oxygenated form of the globins converts NO in nitrate unleashing the NO inhibition of the RC. This restores mitochondrial O_2 consumption and ATP production by oxidative phosphorylation. Under these conditions the globins might favour the delivery of O_2 and/or fatty acids (FA) to the mitochondria. It is further shown that the normoxic condition promotes the activity of the NADPH oxidases (NOXs) with the formation of ROS. The ROS level is suggested to be eventually increased by means of a ROS-induced ROS release (RIRR) mechanism involving the mitochondrial RC. The consequent enhanced pro-oxidative state of the cell functions as a signal inducing the HSPC to differentiate. The protective role of the nuclear Mb under this condition is removed by its cytoplasmic re-localization. Depending on the prevailing conditions, set by extrinsic signals, ROS can also activate under normoxic conditions HIF thereby delaying differentiation of HSPCs if these are mobilized to restore exhausted niches or act at hypoxia-injured tissues. See text for further considerations.

non-small cell lung cancer, simultaneous expression of Mb and Ngb was recently reported (Oleksiewicz *et al.*, 2011).

From the differential O_2-sensitive optical spectra reported in our study, it was possible to estimate an overall content of ≈0.01 nmol haemes per 10^6 HSPC corresponding to ≈180 ng globin per 10^6 HSPC. This value is significantly higher than the upper range reported in tumour samples and cancer-derived cell lines (Flonta *et al.*, 2009; Kristiansen *et al.*, 2010). As the cellular volume of $CD34^{+}$-HSPC is about 400 μm^3 (Sharma *et al.*, 2008), an intracellular concentration of ≈25 μM can be estimated. This value is about one order of magnitude lower than that in muscular cells. However, considering the relatively large nucleus/cytoplasm volume ratio in HSPCs, the cytoplasmic concentration of the globins might be significant.

In myocytes, Mb is widely accepted to function as temporary 'store' for O_2, able to buffer short phases of exercise-induced increases in O_2 flux during which it supplies the gas to the mitochondria (Ordway and Garry, 2004). More controversial, is the role of Mb-mediated facilitated diffusion of O_2 within muscle cells (Wittenberg, 1970; Jürgens *et al.*, 1994). In addition to these canonical activities, Mb has been shown to participate in the NO homeostasis in muscle through either scavenging (Brunori and Gibson, 2001; Flögel

et al., 2001) or producing the NO molecule (Hendgen-Cotta *et al.*, 2008). Further possible functions of Mb in muscle include synthesis of peroxides (Khan *et al.*, 1998), scavenging of ROS (Flögel *et al.*, 2004) and binding of fatty acids (Sriram *et al.*, 2008). Concerning Ngb, its exact physiological role is still debatable. Several proposed functions of Ngb overlap with those of Mb (Burmester and Hankeln, 2009). Ngb was also found to co-localize with mitochondria (Yu *et al.*, 2012). Moreover, Ngb might be involved in a signal transduction pathway, for example, by inhibiting the dissociation of GDP from G protein α or might be part of a redox process that is, for example, instrumental in preventing apoptosis via reduction of cytochrome *c*.

The transcript analysis for Mb expression in HSPCs revealed, in addition to the standard mRNA expressed in muscle, the prevailing occurrence of an alternative transcript recently described in breast cancer cells (Kristiansen *et al.*, 2010). In this alternative Mb mRNA, a distal HRE from the transcription start accounts for the HIF-1/2α-dependent up-regulation of Mb in hypoxic cancer cells (Kristiansen *et al.*, 2011). In the case of Ngb, albeit its promoter apparently lacks the conventional HREs, nevertheless, a number of studies have clearly established that hypoxia and HIF-1 are necessary for the up-regulation of Ngb expression in the nervous system (Haines *et al.*, 2012a,b). Accordingly, we found that the expression of Mb and Ngb are dependent on hypoxia and the stabilization of both HIF-1α and-2α in HSPCs (Piccoli *et al.*, 2007c and unpublished data).

Normal and cancer stem cells share, to a certain extent, similar properties, such as the propensity to survive in hypoxic milieu, a predisposition towards a glycolysis-based metabolism, the ability to maintain a long-lasting quiescent undifferentiated state and to undergo self-renewing divisions (Reya *et al.*, 2001; Li *et al.*, 2006). Therefore, studies aimed at elucidating the role played by members of the globin family in cancer cell biology might provide clues to rationalize the functional role of Mb and Ngb in HSPC. In this context, results from a study wherein Mb-negative human lung carcinoma cells were engineered by lentiviral transfection to ectopically express mouse Mb are noteworthy (Galluzzo *et al.*, 2009). The experimental Mb-expressing tumours displayed reduced or no hypoxia, minimal HIF-1α levels, decreased vessel density, a more differentiated cancer cell phenotype and a largely suppressed local and distal metastatic spreading. Although these beneficial outcomes in Mb-overexpressing tumours might result primarily from the reduction in tumour hypoxia, nevertheless, the relatively high levels of forced ectopic expression of Mb are not comparable with the low, picomolar, concentrations of Mb detected in cancer cells. Intriguingly, knock down of Mb in breast cancer cell lines resulted in an unexpected increase in O_2 uptake and elevated activities of mitochondrial enzymes during hypoxia and silencing of Mb transcription attenuated proliferation rates and the motility of cancer cells under both hypoxic and fully oxygenated conditions (Kristiansen *et al.*, 2011). To note, Cygb, another Mb-related globin detected in lung and breast cancer cells, was shown to exert a tumour-suppressor effect as its silencing in Cygb-positive cells resulted in increased colony formation, whereas induced Cygb expression in Cygb-negative cells reduced colony formation. All together, the evidence available in the literature converge to define a role for Mb as tumour suppressor, as well as for other members of the globin family (Shivapurkar *et al.*, 2008), but seemingly not directly linked to the binding and transport of O_2.

As discussed in the previous paragraph, the notion that hypoxia is a condition preventing oxidative stress is challenged by the repeatedly reported observation that under hypoxia cells produce more ROS than under normal O_2 tension and that the source of such 'hypoxic' ROS is the mitochondrial RC complex III. However, the physiological mechanism of ROS production by the hypoxic RC still needs to be defined. Indeed, the low K_M for O_2 of the cytochrome *c* oxidase sets the environmental O_2 concentration that effectively would limit the RC function to values below 1 μM (i.e. <0.1% pO_2), which is unlikely to be reached in the BM endoblastic niche. Moreover, even if such a severe hypoxic condition occurred, the low concentration of O_2 would limit ROS production, which is, in any case, a second-order reaction with single electron donors. Therefore, other mechanisms might contribute to inhibit cytochrome *c* oxidase and, at the same time, to account for a significant generation of ROS. Among those, the reversible NO inhibition of cytochrome *c* oxidase has been shown to be physiologically relevant in shifting ATP production from OXPHOS to glycolysis (Sarti *et al.*, 2012a,b,c) as well as in eliciting signalling events, including the diversion of O_2 to non-respiratory substrates and the generation of ROS (Erusalimsky and Moncada, 2007).

Combining these caveats and stimulated by our finding of globins in HSPCs, we would like to propose a model whereby Mb/Ngb provides, under hypoxic conditions, a source of NO by their hypoxia-dependent nitrite-reductase activity (Figure 2). NO is a potent competitive inhibitor of cytochrome *c* oxidase, especially under conditions of limited O_2 availability (Sarti *et al.*, 2012a). Thus, inhibition of the terminal part of the mitochondrial RC would elicit ROS formation at the complex III site under non-severe hypoxia conditions. Moreover, NO has also reported to be an inhibitor of complex I (Brown and Borutaite, 2004). Of note, nitrite-reductase activity has also been recently demonstrated for cytochrome *c* oxidase itself with production and auto-inhibition of NO (Ball *et al.*, 2012). However, the low amount of cytochrome *c* oxidase in HSPC as compared with other cell types would be inadequate to guarantee an optimal generation of NO.

The process described above would contribute to the hypoxic stabilization of HIF-1/2α by the mechanism explained earlier, involving ROS- and NO-mediated inactivation of PDHs and FIH-1. The beneficial up-regulation of HIF-1, which, in addition to affecting HSPC metabolism, preserves the HSPC undifferentiated state, needs, however, to be reconciled with the known effect that redox signalling has on the cell cycle. Indeed, the main adaptive response of many cell types to a mild oxidative insult is activation of the cell cycle, enhanced proliferative activity and eventually differentiation (Noble *et al.*, 2005; Burhans and Heintz, 2009; Finkel, 2011; Antico Arciuch *et al.*, 2012; Chiu and Dawes, 2012). This would be detrimental in HSCPs if maintenance of the stemness profile is required. However, it is important to recall that redox signalling operates differentially in distinct intracellular compartments and oxidative modifications of nuclear located transcription factors are emerging as an important mechanism controlling gene expression (Go and Jones, 2010; Lukosz *et al.*, 2010).

In this context, our finding that Mb specifically accumulates in the nucleus of $CD34^{high}$-HSPC might be relevant to the cellular programme of multipotency maintenance. Conceivably, the Mb accumulated in the nucleus is not related to O_2-storage/-facilitated diffusion, but rather suggests its involvement in the scavenging of reactive species to prevent nitro-oxidative modification of DNA or of redox-sensitive transcription of genes participating in the differentiation process (Ogasawara and Zhang, 2009; Owusu-Ansah and Banerjee, 2009; Brigelius-Flohé and Flohé, 2011; Kobayashi and Suda, 2012; Sardina *et al.*, 2012). Interestingly, it has been shown that ROS-mediated oxidation of sensitive cysteine residues in PMLs controls their degradation and nuclear body biogenesis in acute promyelocytic leukaemia and BM progenitor cells (Jeanne *et al.*, 2010). Moreover, Mb might interact directly with stem cell-specific nuclear transcription complexes. A recently reported MS-based analysis revealed a complex interatomic network of Mb with partners known to have antioxidant and anti-apoptotic functions, as well as with a number of nuclear proteins involved in DNA duplication/transcription/repair, chromatin stabilization, ribosomal assembly and nuclear import (Haines *et al.*, 2012a,b). Importantly, many Mb-interactants overlapped with those of Ngb and were modified by hypoxia. Therefore, the nuclear localization of Mb that we observed in $CD34^{high}$-HSPC might serve as a compartmentalized antioxidant buffer or as NF interactants to prevent unwanted activation of specialization genes, thereby preserving quiescence.

An alternative but consistent scenario could be that Ngb acts as a generator of cytoplasmic NO and Mb as a nuclear antioxidant or protein interactant. This would account for the occurrence of two different globins, which act in concert to fine-tune the level and intracellular compartmentalization of reactive species.

Once HSPCs move from the hypoxic osteoblastic niche to a more oxygenated milieu, for example, the vascular niche, this is perceived as an activating signal. Indeed, there is accumulating evidence to support the notion that, even in the absence of specific biochemical signals, changes in environmental physical and chemical parameters may cause the stem cell to egress from its quiescence. Changes in the intracellular redox state seemingly represent a signalling system that anticipates the growth/differentiation factor-mediated commitment and/or makes the stem cell more responsive to specific proliferative/differentiation stimuli. Assuming that this pertains to the $CD34^{low}$-HSPC subtype, the re-localization of Mb thereof from the nucleus to the cytoplasm might be a way to enable reactive species to signal, at the nuclear level, proliferative/differentiation stimuli.

Given the kinetic features discussed previously, NOXs are well-suited to sense and signal by ROS production the change in O_2 concentration when HSPCs move from the periosteal to the vascular niche. This is consistent with the documented primary role of NOX-generated ROS in fostering proliferation/differentiation of HSPCs. However, the contribution of mitochondria to ROS-production cannot be ruled out and the possibility that a 'ROS-induced ROS release' process and/or the shift towards a more active oxidative metabolism exists cannot be ruled out.

It should be noted that the nitrite-reductase activity of globins requires that they are in their deoxygenated form; therefore, under normoxia, a very low, if any, Mb/Ngb-mediated NO production is expected. Instead, both Mb and Ngb function under normoxia as dioxygenases, converting enhanced NOS-mediated NO production to nitrate (Sarti *et al.*, 2012a), thereby unleashing the NO-dependent inhibition of the mitochondrial respiratory activity. This would result in stimulation of oxidative phosphorylation and related ATP production adapting the HSPC bioenergetic metabolism to cope with the larger energy demand required for proliferation/differentiation. Under this more bioenergetically active condition, the cytoplasmic localization of Mb and Ngb might also serve to smooth the intracellular O_2 gradients nearby the peri-mitochondrial space and/or to transfer fatty acids.

Conclusions

Taken together, the growing body of accumulating evidence convincingly indicates that a complex interaction between ROS-mediated signalling and metabolic profile determines the fate of a cell. Stem cells and specifically HSCs are not an exception and exploit redox signalling to optimize the delicate balance between quiescence, self-renewal and commitment. What makes HSCs (and progenitors) different is that they exploit ROS to control both the maintenance of a quiescent condition and activation of self-renewal followed by proliferation and differentiation. This apparent paradox can be explained by the following: (i) the oxidizable targets may have a different sensitivity to ROS, so that a low ROS level would activate pathways suited to promote cell survival by a glycolysis-based metabolism, whereas higher ROS levels would activate different pathways leading to mitochondrial biogenesis and to more oxidative metabolism; (ii) the chemical properties of diverse reactive species (i.e. $O_2^{\bullet-}$, H_2O_2 and $OH^{\bullet}$) are different and affect distinct targets; (iii) the source, stability and localization of ROS can contribute to the compartmentalization of the redox signalling; (iv) the interplay with other reactive species such as NO may add a further level of complexity and robustness of the signal transduction responsiveness; and (v) the cross-talk between upstream and downstream ROS-involving signal transduction pathways may generate negative or positive feedback loops resulting in different adaptive outcomes.

It is important to realize that redox signalling alone may not be sufficient to orchestrate the complex programme that regulates the fate of a stem cell, but rather may set the pre-commitment conditions allowing stem/progenitor cells to be more or less responsive to growth/differentiation factors. In this context, the control of 'mitochondrial differentiation' appears to be a checkpoint step that precedes and influences the stem cell fate.

Importantly, all the above-mentioned pathways are capable of being manipulated by specific pharmacological agonists or antagonists, paving the way to regulate the metabolic signature of HSPCs for future clinical applications.

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Conflict of interest

The authors have no conflicting financial interests.

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