Abstract

Significance: Blood forming, hematopoietic stem cells (HSCs) mostly reside in the bone marrow in a quiescent, nonmotile state via adhesion interactions with stromal cells and macrophages. Quiescent, proliferating, and differentiating stem cells have different metabolism, and accordingly different amounts of intracellular reactive oxygen species (ROS). Importantly, ROS is not just a byproduct of metabolism, but also plays a role in stem cell state and function. Recent Advances: ROS levels are dynamic and reversibly dictate enhanced cycling and myeloid bias in ROS\textsuperscript{high} short-term repopulating stem cells, and ROS\textsuperscript{low} quiescent long-term repopulating stem cells. Low levels of ROS, regulated by intrinsic factors such as cell respiration or nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) activity, or extrinsic factors such as stem cell factor or prostaglandin E2 are required for maintaining stem cell self-renewal. High ROS levels, due to stress and inflammation, induce stem cell differentiation and enhanced motility. Critical Issues: Stem cells need to be protected from high ROS levels to avoid stem cell exhaustion, insufficient host immunity, and leukemic transformation that may occur during chronic inflammation. However, continuous low ROS production will lead to lack of stem cell function and opportunistic infections. Ultimately, balanced ROS levels are crucial for maintaining the small stem cell pool and host immunity, both in homeostasis and during stress situations. Future Directions: Deciphering the signaling pathway of ROS in HSC will provide a better understanding of ROS roles in switching HSC from quiescence to activation and vice versa, and will also shed light on the possible roles of ROS in leukemia initiation and development. Antioxid. Redox Signal. 21, 1605–1619.

Introduction

The blood and host immunity requires a continuous supply of mature leukocytes and red blood cells with a finite lifespan throughout life. This process is enhanced after acute stress situations such as bleeding, infections, or irradiation and is attributed to the unique population of hematopoietic stem cells (HSCs) and progenitor cells. The hematopoietic stem and progenitor cells are a small population of undifferentiated cells that reside in the bone marrow (BM) and can undergo self-renewal by giving rise to mature cells, while maintaining a constant number of the stem cell pool. Another distinct feature of these cells is their ability to migrate out of the BM to the peripheral blood. This process is enhanced on stress situation as a part of host defense and repair mechanisms. In addition, HSCs injected to the blood stream, as done in BM transplantation, can also home to the BM and re-establish the HSC pool as a lifelong reservoir of new blood and immune cells [reviewed in Ref. (50)]. Emerging evidence shows that oxidative stress, in particular reactive oxygen species (ROS) content, influences stem cell migration, development, and self-renewal as well as their cell cycle status.

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ROS are organic and inorganic molecules that have an odd number of electrons in their outer valence shell. ROS refers to O2- free radicals, as well as to nonfree radicals’ derivatives. When molecules are oxidized during metabolism, the oxygen molecule itself is reduced to water, giving rise to intermediate ROS, including hydroxyl radicals (OH·), hydrogen peroxide (H2O2), and superoxide anion radical (O2·−). These molecules are highly reactive due to the presence of unpaired valence shell electrons and can cause a chain reaction between molecules that eventually results in acute oxidative damage. Under normal physiological conditions, ROS can be formed during numerous reactions in vivo, including enzymatic activity, activated phagocytic cells, mitochondrial respiration, and by nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase). These reactions generate ROS as a part of their normal activity [reviewed in Refs. (46, 65)]. Though high levels of ROS may harm cells by inducing DNA damage and promoting apoptosis, moderate levels of ROS are needed for hematopoiesis during embryonic development (25), and they are also required in adult hematopoietic homeostasis. In quiescent stem cells, ROS levels are kept low, thus supporting their long-term repopulation ability. Elevation in ROS content drives stem cell differentiation to short-term repopulating cells and further on to myeloid differentiation as was shown in mouse models (39, 40) as well as in Drosophila (70). Importantly, ROS levels may be reduced by in vitro pretreatment with the ROS inhibitor N-acetyl cysteine (NAC), or with a p38 inhibitor in enriched murine ROC(high) short-term repopulating progenitor cells. This enables the cells to restore their long-term repopulation ability, which is a hallmark of stem cells (39, 40). These preliminary results suggest reversibility of ROS high levels in stem cells; however, direct evidence on a single stem cell level is still lacking. Taken together, we may suggest that the quiescent and cycling state of HSCs involves fluctuations in ROS levels, affecting their motility, proliferation, differentiation, and repopulation potential (Fig. 1).

While exceedingly high ROS levels were shown to promote exhaustion of the stem cell pool in the BM (38, 39), extremely low ROS levels in HSCs, in models where ROS content was reduced below normal levels, showed defects in their differentiation ability, leading to their impaired repopulation capacity (41). Thus, minimal ROS levels are crucial for stem cell function and balanced hematopoiesis.

In the complex structure of the BM, the HSCs are regulated by their adjacent cells in the microenvironment, and by the cytokines, chemokines, and additional lipid effectors that they produce. ROS stand at the cross-point of the regulations exerted on the stem cells, as a signaling molecule as well as the manifestation of their metabolic profile. This review will outline the complex and multi-player regulation of ROS in HSCs, and the function of ROS in the HSCs themselves. We will discuss the multiple roles of ROS in the context of HSC function. We will outline the importance of dynamic ROS signaling for normal differentiation of both hematopoietic and mesenchymal stem cells, as well as motility and self-renewal of HSCs. The review will elaborate on the intrinsic (stem cell derived) as well as the extrinsic (BM stroma and endothelial cells microenvironment derived) regulation of balanced ROS levels to maintain a constant functional stem cell pool and maturing leukocytes in the BM reservoir. Moreover, ROS also plays an important function in inflammation and cancer propagation. Thus, we will also discuss how unbalanced ROS levels can hamper stem cell motility or proliferation and differentiation in chronic granulomatous disease (CGD)-induced immune deficiency or leukemia development, respectively.

Microenvironmental Regulation of HSC ROS Content

Stem cells reside in the BM in distinct microenvironments, which provide a milieu to support and maintain the primitive cells in a quiescent mode. Under stress conditions, undifferentiated progenitor cells can be triggered by their microenvironment to undergo enhanced proliferation and differentiation to withstand the increased demand of the immune and hematopoietic systems for new leukocytes and blood cells. The progenitor cells are found in the vicinity of BM stromal (5, 14, 59, 95, 121), nervous (117), and myeloid, hematopoietic (7, 9, 55) cells, all acting together by producing chemokines, lipid factors, and adrenergic signals (92) to maintain a functioning pool of precursor cells.

The endosteal and perivascular bone areas are mostly discussed as the morphological locations of stem cells in the BM. These locations are essentially found between the calcified bone region and the sinuses, which are a part of the BM vasculature. In the endosteal region, near the calcified bone margins, osteoblast precursors were previously shown to contact and support HSCs (5, 121). Recently, reports identified the quiescent HSCs near the sinuses of the BM, in a distance of approximately 5-cell diameter. Moreover, these studies suggested that the endosteal surface homes the more committed lymphoid progenitors, and that osteoblasts contribute to HSCs maintenance to a lesser degree than the cells of the perivascular microenvironment (13, 23, 44). The area near the sinuses, termed the perivascular niche, is home to the stromal reticular cells such as CXCL12 abundant reticular (CAR) cells (23, 95), Nestin-GFP+ mesenchymal stromal precursor cells (59), and immature Leptin receptor+ perivascular cells (14). These cells were found in contact with primitive SLAM+ HSCs, and provide essential cytokines such as the chemokine CXCL12, which maintains stem cell

![FIG. 1. Intracellular reactive oxygen species (ROS) regulate self renewal versus activation of hematopoietic stem cells (HSCs). Quiescent, long-term repopulating stem cells are characterized by low levels of ROS. Elevation of ROS levels in the stem cells would enhance cycling and motility of the stem cells and provide short-term repopulation ability. The high levels of ROS are reversible and reduction of ROS levels in the stem cell would induce reduced cycling and restore long-term repopulation potential. Excess of ROS, on the other hand, would lead to senescence and apoptosis that cannot be rescued.](image-url)
quiescence and self-renewal and stem cell factor (SCF) that is essential for their proliferation in the BM.

HSCs were suggested to reside in areas of low perfusion, based on perfusion of Hoechst33342 in the murine BM, which mimics oxygen and nutrient transport (72). These experiments showed that the long-term repopulating stem cells incorporated the lowest amount of Hoechst33342 (72, 115), suggesting that they are found in the low perfusion areas. Furthermore, the Hoechst-low cells were shown to be in a hypoxic state (72) and are less cycling (115), leading to the suggested notion that the quiescent long-term repopulating HSCs are maintained in hypoxic conditions. A biophysical model simulating PO2 in BM showed that a distance of only a few cells from the blood flow causes a major reduction in PO2 levels which can be sensed by the cell (8). This was confirmed by direct measurements of PO2 in the BM of mice, revealing that hypoxic conditions exist in perivascular areas (91a). This suggests that even at a 5-cell distance from a blood sinus, as was described in the perivascular area, the HSC may experience hypoxic conditions. Recently, morphological-anatomical imaging of primitive hematopoietic cells showed that they reside in a high frequency near the sinusoids adjacent to the endostal area and are stained for pimonidazole and hypoxia-inducible factor 1-α (HIF-1α), suggesting that the HSCs are in a hypoxic state (67). However, HIF-1α is activated by many cytokines in addition to lack of oxygen (112).

Hypoxia, characterized by low O2 availability, can influence cells via the hypoxia-inducible factors (HIF) protein family, though independent hypoxic effects, such as glutamate receptor trafficking, were noted in Caenorhabditis elegans (71), and were reported but remained poorly understood. In HSCs, hypoxia activates signaling transduction machinery acting via HIF proteins, which dictate cellular metabolism. In the hematopoietic system, HIF-1 and HIF-3 were shown to be expressed in HSCs (96), while HIF-2 was shown to be mainly involved in erythropoiesis (86). The role of HIF-3 is uncertain in the primitive hematopoietic system, but it was shown to be a transcriptional target for HIF-1α (98), which was demonstrated to play a major role in stem cell regulation. Activation of HIF-1α would shift cellular metabolism to glycolysis rather than mitochondrial respiration, thus limiting ROS generation (113). In addition, hypoxia was also shown to reduce NADPH oxidase activity and, consequently, ROS production. Thus, hypoxia may influence both major generators of ROS in the cell mitochondria and NADPH oxidases. However, HIF-1α can be activated by cytokines independently of hypoxia, such as interleukin 1β (IL-1β) and tissue necrosis factor (TNFα) (112), suggesting that ROS levels may vary when hypoxia is not altered. The low nutrient and hypoxic state would affect both the metabolic activity and thus ROS production by the progenitor cells. Interestingly, the cells in the BM microenvironment participate in the regulation of ROS levels in stem cells, to control and balance ROS content in primitive cells (Fig. 2).

Mesenchymal stromal cells (MSCs) were shown to import ROS from stem cells to the stromal cells via connexin gap junctions, and by that to reduce ROS content in primitive cells (99). This was done via Connexin-43 gap junctions, which were shown to be highly expressed by immature MSCs (59, 83). In addition, prostaglandin E2 (PGE2) secreted from α-smooth muscle actin + macrophages maintain low ROS levels in stem cells by inhibiting Akt phosphorylation (55). Together, the supporting cells contacting primitive cells serve to reduce ROS levels in stem cells, attenuating excessive proliferation and differentiation that would induce stem cell pool exhaustion. Nevertheless, the progenitor cells themselves are able to cope with hypoxic conditions due to several intrinsic mechanisms that include transcription factors and their metabolic state.

The Metabolic States of Quiescent and Cycling HSCs

In steady state, about 70% of the cells comprising the stem and progenitor cell pool in the BM were shown to be in a low cycling quiescent mode (114). These dormant, quiescent cells can switch to cycling cells, while still remaining in a stem cell state (114). Moreover, this study suggested that cycling stem cells can reverse from cycling back to a dormant state (114). Since cycling and quiescent stem cells have different energy requirements, it is possible that this reversible switch between cycling and dormancy also involves metabolic alterations and, concomitantly, fluctuations in ROS levels.

Nutrient and oxygen deficiency would dictate a state of cell quiescence, a period when the energy consuming activities of the cells are on hold. It was recently shown that an enzyme family mediating anaerobic glycolysis, Pdk, also regulates precursor cell cycle (97). Quiescent stem cells with long-term repopulating capacity were shown to express high levels of Pdk2 and Pdk4, mediated by HIF-1α, which promoted anaerobic glycolysis at the expense of mitochondrial respiration (97). Thus noncycling primitive cells use anaerobic glycolysis for energy production. The immature cell pool rejuvenates itself by asymmetric divisions, which were recently suggested to rely on the nutrient-sensing peroxisome

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**FIG. 2. Environmental factors maintain low ROS levels in the stem cells to maintain the stem cell pool.** The mesenchymal stromal cells and α-smooth muscle actin (αSMA) + macrophages act together to preserve low ROS levels in HSCs. The αSMA + macrophage contains cyclooxygenase-2 (COX-2) that produces prostaglandin E2 (PGE2) (1), which is secreted to the vicinity of the stem cell (2) and reduces its intracellular ROS levels. PGE2 also promotes CXCL12 production from the mesenchymal stromal cell (3). In addition, the stem cells are connected to the mesenchymal stromal cells via connexin gap junctions that transfer ROS from the stem cell to the mesenchymal stem cell (4) and contribute to the reduction of ROS levels in the stem cell.
proliferator-activated receptor δ (PPARδ), which activates fatty acid oxidation in the mitochondria (37). By regulating asymmetric divisions in the stem cell compartment, PPARδ was found to maintain a pool of undifferentiated cells and prevent stem cell exhaustion (37). However, the main role of primitive cells is to provide mature hematopoietic cells by undergoing proliferation and differentiation. For these functions, it was shown that stem and progenitor cells can switch their metabolic state to mitochondrial respiration. Under mitochondrial respiration, progenitor cells generate more ATP, which is required for the energy-consuming tasks of their proliferation, differentiation, and motility. Without protein tyrosine phosphatase, mitochondrial-1 (PTPMT1) activity, a PTEN-like phosphatase located in the cell mitochondria involved in mitochondrial oxidative phosphorylation, stem cells lose their differentiation ability. This ultimately causes repopulation failure, as the stem cells are unable to give rise to mature functioning blood and immune cells (118). Additional evidence for mitochondrial derived ROS involvement in stem cell proliferation comes from mice lacking tuberous sclerosis-1 (Tsc1). Tsc1 inhibits mammalian target of rapamycin (mTOR), which is a key regulator in cell metabolism. Interference with the Tsc1-mTOR pathway was shown to elevate HSC cycling and mobilization, and it hampered their long-term repopulation potential (18). Moreover, Tsc1 deletion in mice caused increased mTOR activity, elevated mitochondrion biogenesis accompanied by elevated ROS levels (6). This was accompanied by loss of self-renewal capacity of the HSCs and hematopoiesis failure, which could be corrected by reducing ROS levels.

Generation of ROS is inevitably linked with cell respiration. While anaerobic glycolysis produces only low ROS levels, mitochondrial respiration creates much higher ROS levels. While excess of ROS levels result in senescence and cell death, low ROS levels are crucial for maintenance of the stem cell pool. Higher redox signaling is needed for their increased energy-consuming activities such as proliferation, differentiation, and migration. Increased stem cell quiescence was also noted in Akt1/2-deficient mice, similar to mice over-expressing HIF-1α. Akt1/2 are transcription factors modulating cell metabolism, and their loss resulted in reduced motility and increased quiescence of primitive cells, resulting in impaired long-term repopulation ability concomitant with reduced ROS levels (41). Importantly, forced elevation of ROS levels rescued the differentiation block (due to over-quiescence), and restored the ability of the Akt1/2 knockout stem cells to give rise to differentiated progenies that durably repopulated the blood and immune systems of the transplanted mice (41). Interestingly, reductions in HIF-1α also led to increased, high ROS levels and loss of the stem cell pool, while over-stabilization of HIF-1α resulted in reduced low ROS levels, below the needed threshold for adequate stem cell function and their self-renewal (96).

**Cell Intrinsic Factors Mediating ROS Levels in HSCs**

Long- and short-term repopulating HSPC can be discriminated by their ROS levels (40). Primitive cells with low ROS levels presented superior long-term repopulation capacity compared with high ROS progenitor cells, which mostly presented short-term repopulation capacity and a tendency toward myeloid differentiation (40). Moreover, ROS levels are dynamic and reversible, and stem cells can switch from ROS\textsuperscript{high} back to ROS\textsuperscript{low} and vice versa, changing their phenotype and function accordingly (40) (Fig. 1). Indeed, limiting ROS content in vivo prevents exhaustion of the stem cell pool (38). Recently, different factors were noted to regulate ROS levels in immature cells [reviewed in Ref. (106)] (Fig. 3). One of the major factors that preserves stem cells is HIF-1α (16, 97) (Fig. 4). HIF-1α is a transcription factor that is not only negatively regulated by oxygen, but also positively regulated by multiple cytokines and chemokines (112), which may play a role in stem cell proliferation, differentiation, and growth. Importantly, HIF-1α by itself induces reduction of intracellular ROS levels (94). HIF-1α is stabilized in stem cells in steady-state conditions and is crucial for maintaining their long-term repopulation ability and preventing their senescence cell death (96). Without HIF-1α, HSCs lose their ROS regulation ability, convert to high ROS levels, and lack hallmark stem cell characteristics of quiescence and long-term repopulation potential (16, 96). However, over-stabilization of HIF-1α also led to impaired reconstitution and loss of stem cell function. Over-
stabilization of HIF-1α caused over-quiescence and impaired motility (96). Together, the stem cells could not migrate to the BM or differentiate and expand the primitive cell pool in transplanted mice to normal size required for adequate function. In steady state, ataxia telangiectasia mutated (ATM), a stress-induced DNA damage protector, is needed to maintain stem cell self-renewal and quiescence by limiting ROS levels in the HSCs and by that preventing HSCs senescence which would be induced by activation of their p16/Retinoblastoma gene in an ROS-dependent manner (38) (Fig. 3). Recently, another link between ROS and ATM was discovered that involves the apoptosis machinery (Fig. 3). It was shown that during homeostasis, ATM keeps low levels of ROS in stem and progenitor cells via phosphorylation and inhibition of BID (a BH3-only BCL-2 family member) (57). However, during DNA damage situations, there is a loss of BID phosphorylation that directs it toward the mitochondria and results in massive ROS production (57) (Fig. 3).

Signaling cascades, including forkhead box protein O (FOXO) (102), specifically FOXO3a (61), are also required to maintain low ROS levels in precursor cells and prevent primitive cell pool exhaustion (Fig. 3). FOXO3a acts upstream to inhibit Akt, and Akt as well as mTOR were shown to promote granulocyte colony stimulating factor (G-CSF)-induced mobilization of stem and progenitor cells in an ROS-dependent manner (22, 100) (Fig. 3). Indeed, mTOR was shown to induce elevation in ROS levels in HSCs that caused increased cycling and mobilization of stem cells (18). Recently, FOXO3a was also found to initiate autophagy in stem cells (64, 111). Autophagy is a self-eating process by which eukaryotic cells degrade and recycle macromolecules and organelles, delivering them into the lysosomes (26). Autophagy protects cells from malignant transformation, by preventing cellular damage, which can also occur due to metabolic stress, when nutrients are limited (64, 111). This is achieved by the removal of toxic proteins and damaged mitochondria, which are the major source of ROS generation. Autophagy relies on oxidative conditions and is triggered by ROS. Nutrient starvation triggers PI3K, which promotes Atg8 lipidation, which is necessary for autophagy (84). Atg8 lipidation is further enhanced by ROS accumulation, which inhibits Atg4, which if not inhibited would promote Atg8 delipidation (84). It was shown that autophagy is activated in HSCs after cytokine withdrawal and without it, HSCs are lost (111). Lack of autophagy caused by Atg7 deletion in mice was shown to result in accumulation of progenitor cells with multiple mitochondria, which led to increased ROS levels in undifferentiated cells and ultimately, to the expansion of progenitor populations in the BM in a manner that resembled acute myeloid leukemia (AML) (64).

FIG. 4. HIF-1α and ROS levels are mediated by steady state versus stress conditions and modulate stem cell dormancy versus proliferation. In steady state, HIF-1α levels in the HSC are high [1] and ROS levels are low. In this state, the HSC are dormant. However, what induces the high expression of HIF-1α is not clear. Possible factors that may induce high levels of HIF-1α are hypoxia and cytokines. Under stress conditions [2], enhanced mitochondrial respiration and NADPH oxidase activity elevate ROS levels in the HSC, concomitant with a reduction in HIF-1α levels. At this stage, HSCs enter cell cycle and proliferation. [3] How ROS levels affect HIF-1α levels in the HSC is yet to be determined. NADPH oxidase, nicotinamide adenine dinucleotide phosphate-oxidase.

As discussed earlier, primitive cells utilize several intrinsic mechanisms that balance their intracellular ROS levels which are based on their tasks and demands. Nevertheless, extrinsic factors such as cytokines provided by the surrounding microenvironment also take part in ROS regulation and finally modulate the quiescent versus active stem cell state.

Extrinsic Factors Modulate ROS Levels in HSCs, Dictating Their Quiescence or Activation

ROS levels are also influenced by a wide range of cytokines secreted from stromal supporting cells in the BM, which promote stem cell self renewal, proliferation, differentiation, and migration (Fig. 5). One of the major players in maintenance of primitive cells is the cytokine SCF that promotes ROS reduction via signaling through its receptor c-Kit, as was demonstrated in fibroblast growth factor-2 (FGF-2)-mediated stem and progenitor cell expansion (36). Interestingly, stem cell expansion by FGF-2 is manifested by elevated long-term repopulating HSCs in the BM (36, 123), without their egress to the blood stream (34). While FGF-2 expanded low ROS stem and progenitor cells in wild-type mice, it induced elevated ROS high hematopoietic progenitors in Wv/Wv mice, harboring mutated c-Kit receptor with defective signaling, resulting in less long-term repopulating stem cells. Thus, SCF-c-Kit signaling is needed for stem cell maintenance (14) by reducing ROS levels in undifferentiated cells to maintain their primitive phenotype.

PGE2 was recently shown to elevate CXCR4 expression on human primitive hematopoietic cells derived from cord blood (21). Since CXCR4 is essential for stem cell maintenance, this suggested the role of PGE2 in stem cell maintenance. Later, PGE2 was shown to preserve long-term repopulating stem cells in culture (29) by stabilizing β-catenin (19, 29). PGE2 was also shown to protect stem cells from apoptosis and to promote recovery of stem cells after myeloablation (75). Cyclooxygenase-2 (COX-2), the enzyme mediating PGE2 production, which is highly expressed by activated monocytes and macrophages, was shown to be essential for maintaining a normal size of the BM stem cell pool in vivo in an AKT-dependent manner (55). PGE2 was also previously shown to promote activation of AKT via its other receptor EP2 in states of inflammation, noted by elevated pAKT levels (54). However, PGE2 was also shown to reduce AKT activity by signaling through the receptor EP4, exerting anti-inflammatory effects (88). Interestingly, EP4 knockout mice presented reduced HSCs content in the BM, while EP2 knockout mice presented normal hematopoiesis, suggesting...
that EP4 is the major receptor for PGE2-mediated regulation on HSC (32). When examined in vitro and in vivo, PGE2 was found to reduce ROS levels in stem cells, via reduction of AKT phosphorylation, maintaining the ROSlow long-term repopulating stem cell pool (55). Accordingly, in COX-2-deficient mice, primitive cells showed higher ROS levels in steady state compared with wild-type mice, suggesting that they have reduced levels of long-term repopulating stem cells. ROS levels were shown to regulate β-catenin levels in cell lines (17, 89). High ROS levels were shown to downregulate β-catenin accumulation (89); however, ROS at low levels induced β-catenin accumulation in cell lines for a period of approximately 1 h (17). Taken together, these results suggest that PGE2-mediated conservation of low ROS levels in stem cells also contributes to the preservation of β-catenin levels in them, thus mediating controlled β-catenin stabilization needed for stem cell self-renewal; whereas high ROS levels promote stem cell loss by accelerated differentiation.

CXCL12 is a cytokine that is essential for stem cell quiescence. It is produced and secreted by many different stromal cell types, including osteoblasts, endothelial cells, and reticular mesenchymal stem and progenitor cells. Cell surface, membrane-bound via heparin sulfate (2, 66, 95, 104) CXCL12 is essential for stem cell quiescence, retention, and self-renewal when presented by the BM stroma. On its secretion and release to the peripheral blood, it induces active stem and progenitor cell migration and mobilization that is elevated by ROS, JNK MMP9, etc. (51, 74). PGE2 stimulation at concentrations that preserve HSCs was shown to elevate membrane-bound CXCL12 expression on BM MSCs (55). However, under terms of stem cell mobilization, ROS signaling by sphingosine 1-phosphate was shown to induce CXCL12 secretion by MSCs in the murine BM (22). Since ROS levels are reduced in HSCs under these conditions, we may assume that ROS levels in MSCs are not high. However, under terms of stem cell mobilization and CXCL12 secretion, we may assume that ROS levels are high. Taken together, this suggests that low levels of ROS promote HSCs retention and quiescence not only by acting directly on HSCs, but also by mediating CXCL12 presentation on the MSCs membrane. On the other hand, elevated ROS levels can promote stem cell mobilization by promoting CXCL12 secretion rather than membrane-bound expression from the MSCs. Oxidative stress further regulates CXCL12 effects by also affecting its major receptor, CXCR4. Nuclear factor (erythroid-derived 2)-related factor 2 (Nrf2), an oxidative stress regulator, was shown to elevate CXCR4 expression in progenitor cells by acting directly on the CXCR4 promoter (103). Without Nrf2, these cells presented increased cycling and proliferation along with hampered migration and homing ability (103), revealing that CXCL12-CXCR4 interactions were hampered. Thus, during oxidative stress, high ROS levels would promote CXCL12 secretion from the stromal cells and elevate CXCR4 expression on HSCs, rendering them more CXCL2 stimulated, perhaps acting together to stimulate HSCs mobilization. Though the hallmark of Nrf2 is ROS detoxification, the effect of Nrf2 on BM HSCs survival and engraftment was shown to be ROS independent (60), emphasizing the broad range effect of oxidative stress. It is currently unknown how CXCL12 would regulate ROS levels in a dose-dependent manner. We suggest that high CXCL12 levels, as present in the BM stem cell microenvironment by stromal cells, promote ROSlow stem cell quiescence and retention. Moreover, low CXCL12 levels during G-CSF induced mobilization, and stress due to enhanced proteolytic enzyme activity and degradation of osteoblasts promoted ROShigh-enhanced stem cell proliferation, myeloid differentiation, and migration potential. However, steady-state CXCL12-CXCR4 interactions are essential to maintain the stem cells in a quiescent nonmotile, ROSlow mode, suggesting that CXCL12 signaling can limit ROS levels. Indeed, loss of membrane-bound CXCL12 in the murine BM results in increased cell cycle and stem cell mobilization (78). Moreover, conditional deletion of stromal CXCL12 expression or its major receptor CXCR4 in adult mice induced hyper proliferation and cycling of HSCs, loss of their quiescence, and, consequently, their long-term repopulation potential, similar to the effects
of elevated ROS levels (66, 78, 95, 104). Interestingly, deletion of CXCR4 and stem cell hyperproliferation was accompanied by a drastic reduction of CD34 expression on the stem cells, a marker indicating proliferation of stem cells (66). Moreover, it might be that high levels of membrane-bound CXCL12 binding to CXCR4 \(^+\) stem cells act to reduce ROS intracellular levels and quiescence induction in BM stem cells. During stem cell mobilization, CXCL12 is released to the circulation, breaking CXCL12 binding to CXCR4 \(^+\) stem cells and promoting elevation of ROS levels in them. In addition, under these conditions, low levels of CXCL12 act as a chemokine promoting stem cell mobilization both by chemotaxis effects and perhaps also by elevation of intracellular ROS levels. Interestingly, an inducible CXCL12 deletion model highlighted two additional environmental factors that could contribute to the elevation in ROS levels due to lack of CXCL12 (104). Tzeng et al. showed that when CXCL12 was deleted, the stem cells translocated from the BM endosteal area to areas around the sinuses (104). The high perfusion area of the sinuses may promote ROS production in the stem cells because of the elevated oxygen levels in the microenvironment. Second, loss of CXCL12 resulted in reduction of the cytokine SCF. Since SCF receptor, c-Kit, signaling was shown to reduce ROS levels in HSCs (36), it might be that loss of SCF also leads to elevation in ROS levels.

Sphingosine 1-phosphate (SIP), a bioactive lipid found in high levels in the blood due to red blood cells, was found to promote stem and progenitor cell egress from the BM during G-CSF and AMD3100-induced mobilization. CXCL12 secretion, mediated by SIP, from MSCs was shown to be inhibited by an ROS scavenger (22). Moreover, SIP elevated ROS levels in immature cells and acted via elevation of Akt signaling (which is attenuated by PGE2) to promote stem and progenitor cell mobilization (22). In addition, intrinsic ROS signaling in undifferentiated cells is needed for their mobilization. Signaling of c-Met, which is dynamically expressed on HSCs, participates in G-CSF-induced mobilization and the ligand for c-Met, hepatocyte growth factor (HGF), can by itself induce HSC mobilization. c-Met is regulated by HIF-1\(\alpha\), and elevates ROS levels in stem cells (15) and G-CSF was shown by itself to elevate ROS levels in hematopoietic stem and progenitor cells (100). Moreover, G-CSF as well as HGF induced mobilization was attenuated when an ROS inhibitor was introduced (100), indicating that ROS signaling was needed for enhanced stem and progenitor cell motility. Interestingly, during stem cell mobilization, the hypoxic areas in the BM were shown to expand, which elevated HIF-1\(\alpha\) levels throughout the BM (53). Moreover, in this study, mobilized cells were found to be hypoxic, indicating that hypoxia can favor motility of hematopoietic stem and progenitor cells. Myeloid progenitor expansion was suggested to deplete the BM from oxygen after G-CSF treatment (53). In addition, G-CSF was shown to promote ROS production in neutrophils and administration of ROS inhibitors along with G-CSF reduced BM osteoblast apoptosis in addition to a reduction in stem and progenitor cell mobilization (91). This study suggests that ROS generated by neutrophils after G-CSF disturbed stem and progenitor cell adhesion to BM stromal cells, as it promoted osteoblast apoptosis (91). However, ROS also affect HSCs directly as a signaling molecule that promotes activation of HSC differentiation and enhanced migration.

The signaling pathway by which ROS promote HSCs mobilization is not yet known. ROS generation is also involved in endothelial cell motility. Moreover, ROS was suggested to be involved in actin reorganization, which allows cell motility [reviewed in Ref. (62)]. Recently, glycogen synthase kinase 3\(\beta\) (GSK3\(\beta\)) was shown to promote primitive hematopoietic cell mobilization via actin reorganization (49). GSK3\(\beta\) is a kinase with multiple intracellular functions, among which is glycogen synthesis. In epithelial cells, GSK3 phosphorylation was shown to be upstream to ROS generation (4). It might be that ROS also promotes stem cell motility by mediating actin rearrangement.

Another means by which ROS may contribute to HSC mobilization is through enhancing matrix-metalloproteinases (MMPs) and other proteolytic enzymes released from myeloid cells in the BM, which participate in G-CSF-induced stem cell mobilization (27, 107). Neutrophils are the main source of MMP-9 in the BM and MMP, including MMP-9, are activated by either serine proteases or ROS (58, 73, 119). In line with this, hind limb ischemia induced postischemic mobilization of BM-derived cells, which was dependent on ROS production by NADPH oxidase-2 (Nox-2), an NADPH oxidase, in Gr-1 \(^+\) myeloid cells accompanied by increased MMP-9 expression (105). In contrast to wild type, Nox-2-deficient mice lacked elevated ROS production and MMP-9 expression on lepton-induced vascular progenitor mobilization. Moreover, administration of cyclophosphamide and G-CSF produced reduced enrichment of HSPC in the peripheral blood of Nox-2-deficient mice compared with wild type (85).

Taken together, these results reveal a cross-talk between ROS and enzyme activity that promotes stem cell mobilization.

**ROS Mediates Proliferation and Differentiation of BM Mesenchymal Progenitor Cells**

BM stromal cells share the hypoxic niches with the hematopoietic progenitor cells that they support. In vitro studies suggest that stromal cells are able to cope with increasing levels of ROS by several mechanisms which mediate their intracellular ROS levels. Similar to HSCs, FGF-2 may promote low ROS levels in MSCs. Culture of MSCs requires FGF-2 to inhibit cellular senescence and maintain their stemness (10). Since high ROS levels promote cell senescence while low ROS levels promote stemness in hematopoietic primitive cells, it is likely that FGF-2 acts on mesenchymal stem cells and reduces ROS levels. In mesenchymal cells, the FGF-2 effect described earlier was shown to be mediated via \(\pi\)PI3K/AKT-Mdm pathway. Mouse double minute (Mdm) was shown to reduce ROS levels in hematopoietic cells and protect them from cell death (1). The involvement of Mdm in MSCs ROS reduction might occur in a similar manner.

Another protecting mechanism is the expression of ROS scavengers. Human stromal cells express high basal levels of the ROS scavenger Glutathione, which enables them to maintain low intracellular ROS levels on introducing high levels of ROS to their culture (108). In addition, high-density lipoprotein was shown to protect BM stromal cells from ROS-induced apoptosis by inhibiting ROS accumulation in...
them in an Akt/PI3K-dependent manner (116). However, accumulation of moderate levels of ROS is important for immature stromal cell differentiation.

The stromal precursors that support stem cells in the BM were shown to have the ability to differentiate to osteoblasts or adipocytes (59, 68, 79). Interestingly, different levels of ROS dictate osteoblast versus adipocyte differentiation of these cells. Intracellular ROS generated by NADPH oxidase 4 in immature stromal cells drives them toward adipocyte differentiation by regulating transcription factors which are important for adipocyte differentiation such as CREB and C/EBPβ that act together with PPARγ, which were found downstream to ROS (42). On the other hand, heme oxygenase-1 (HO-1) activity involves reduction of ROS levels in immature stromal cells by promoting osteoblast differentiation. HO are enzymes that degrade heme into carbon monoxide (CO), iron, and biliverdin, which is a strong antioxidant. HO activity results in reduction of intracellular ROS levels. In immature stromal cells, HO-1, the inducible form of HO, promotes osteoblast differentiation both by inhibiting PPARγ, which is essential for adipocyte differentiation, and by reducing ROS levels, which would otherwise inhibit factors that are important for osteoblast differentiation [reviewed in Ref. (109)]. Moreover, osteoblast differentiation at the expense of adipocyte differentiation was shown to be HIF-1α mediated in human stromal precursors (110). Taken together, we suggest the following: In the BM, immature stromal cells are able to retain their undifferentiated state and maintain their role in supporting primitive hematopoietic cells due to low ROS levels (also in the hematopoietic cells). However, oxidative stress involving elevation of ROS levels will promote proliferation and differentiation of immature stromal cells. Since HIF-1α and HIF-1z are affected by the metabolic state of undifferentiated stromal cells, we might speculate that some immature stromal cells which are found close to the endosteum region are also found in a lower oxygen environment. Thus, they might have low intracellular ROS content and stabilized HIF-1α, which makes them more prone to osteoblast differentiation. On the other hand, stromal precursors, which are found close to the blood sinuses, are subjected to higher levels of oxygen and also produce higher levels of ROS, which makes them more prone to adipocyte differentiation. Thus, the tendency of immature stromal cells toward a specific differentiation lineage might depend on ROS levels and on their location in the BM.

Though regulated levels of ROS play a role in stromal cell function, very high levels of ROS would lead stromal precursors toward senescence or apoptosis (120). In addition, high levels of ROS secreted in the vicinity of BM stromal cells were suggested to promote osteoblast apoptosis by playing a role in G-CSF-induced stem cell mobilization (91).

ROS in Inflammation

In addition to its roles in self-renewal and steady-state preservation of stem cells, ROS also plays a pivotal role in innate immunity acting as signaling molecules, and as a direct effector that kills pathogens on phagocytosis. The ROS-producing NADPH oxidase Nox-2 is a target gene of HIF-1α, and, in turn, Nox-2-derived ROS promote HIF-1α expression (12, 106). Though its cross-talk with HIF-1α suggests its role in stem cell self renewal, Nox-2 activity attributes to the phagocytosis ability of myeloid cells. Nox-2 reduces molecular oxygen to superoxide anions in the lumen of phagosomes, triggering a cascade of additional ROS derivatives. Their creation, called “respiratory burst,” facilitates the killing of engulfed pathogens, and it is involved in the activation of other antimicrobial mechanisms such as formation of neutrophil extracellular traps and activation of proteases from granules (3, 48). In addition to its role in foreign antigen elimination, NADPH oxidase complexes also control the extent of the immune response. Phagosome-derived ROS are able to permeabilize membranes and activate the redox-sensitive transcription factor Nrf2, which limits inflammatory responses (77).

CGD is a prototype for a pathological state in which the respiratory burst in phagocytes is defective. This rare, inherited myeloid disorder is caused by either Nox-2 deficiency or mutations in the NADPH oxidase complex subunits. The disease is manifested by sterile hyper-inflammatory (11, 77) and the inability of mature phagocytes to kill ingested microorganisms. This eventually manifests in severe and life-threatening granuloma and abscess formation and despite treatment with antibiotics, the patients usually die at a young age. Nowadays, CGD treatment by gene therapy approach is examined, relying on correction of the ROS production defect in CD34+ hematopoietic progenitors. Being immature hematopoietic cells, the corrected cells are transplanted back to the patient and are expected to repopulate the patient’s immune system with normally functional hematopoietic cells. However, one of the major problems in this therapy is that usually only a short-term engraftment occurs. When long-term engraftment is achieved, it is accompanied by a myelodysplastic syndrome (MDS), suggesting that the genetic manipulation hampers long-term repopulation and proliferation control in the manipulated stem and progenitor cells [as reviewed in Ref. (24)]. These phenomena, that is, short-term repopulation and enhanced proliferation and differentiation, characterized primitive hematopoietic cells with high ROS levels, suggesting that perhaps the correction which elevates ROS production might also hamper the control of ROS levels. Interestingly, transplantation of healthy donor CD34+ progenitor cells is successful in CGD patients, indicating that the defect is stem cell intrinsic is not influenced by the BM microenvironment (101), and again suggesting that internal regulation of ROS levels in the stem cells is hampered. However, Nox-2 deficiency as may be noted in CGD patients can also influence stem cell mobilization. In CGD patients, G-CSF-induced stem and progenitor cell mobilization is reduced (87). ROS promotes monocyte motility (45) and regulates neutrophil migration via actin polymerization (81), which is needed for the egress of stem and progenitor cells (76). Taken together, Nox-2 deficiency or its reconstitution seem to be involved in determining the engraftment potential of mobilized progenitor cells, and phagocytic ROS appears to play a crucial role in the mobilization and motility of myeloid cells.

Although no toxic effects have been described so far, over-expression of the transgene gpr1phox, which recapitulates ROS production, might induce “leaky” ROS production in stem cells. This elevation in ROS content might hamper their long-term repopulation capacity. However, this could not be
confirmed experimentally and insertional activation of proto-oncogenes by retroviral vector was identified as the MDS promoting force (69, 93).

ROS in Hematopoietic Malignancies

ROS can be involved in the initiation and progression of hematopoietic malignancies such as MDS and AML either by inducing unspecific oxidative damage of DNA, lipids, and proteins or by hyperactivation of ROS signaling pathways (46, 56). Chronic oxidative stress is observed in a variety of tumors of different tissue origin and can also induce changes in nonmalignant bystander cells in a paracrine manner due to the freely membrane-permeable nature of some ROS such as H$_2$O$_2$ (30).

Sorted human leukemic sub-populations transplanted into immune-deficient mice were shown to functionally initiate leukemias in the chimeric mice, pointing at the existence of leukemic stem cells (52). Recently, it was shown that some human leukemic stem cells are characterized as ROS$^{low}$ and over-express BCL-2 (47). In normal stem cells, interference with the localization of the BCL-2 family member BID to the mitochondria kept ROS levels low, by limiting mitochondrial ROS generation (57). However, leukemic stem cells seem to depend on mitochondrial respiration, as inhibition of BCL-2 in the human leukemic cells hampered mitochondrial respiration, which could not be compensated by glycolysis that is used by the normal stem cells (47). Importantly, inhibition of BCL-2 targeted specifically human leukemic stem cells and promoted their eradication (20, 47). In addition to BCL-2, glutathione peroxidase 3 (Gpx3), an ROS scavenger, was shown to correlate with a high frequency of leukemic stem cells (28). Interestingly, over-expression of Gpx3 did not affect clonogenic proliferation of leukemic progenitor cells, but leukemic stem cells over-expressing Gpx3 presented elevated ability to repopulate the BM (28). This study suggested that the ROS scavenger Gpx3 maintains the stem cell characteristics of both normal and leukemic stem cells (28).

Concluding Remarks

The hematopoietic system is regulated by a variety of supporting cells, chemokine, cytokines, and lipid effectors that mediate self-renewal as well as by proliferation, differentiation, and motility of stem and progenitor cells. At the junction of these regulations, ROS seems to play an important role. Energy consumption underlies all cell functions, and the rate of energy production should be controlled to achieve enough fuel for cell functions without over production of hazardous byproducts such as ROS. Interestingly, ROS released during energy production serves by itself as signaling molecules, working in a window of low to moderate levels to mediate self renewal versus proliferation and differentiation of stem cells. Thus, modulation of energy production also serves to dictate cell function not only by limiting or elevating the fuel needed for the cells, but also by limiting or elevating ROS signaling. Not surprisingly, a variety of transcription factors that were found crucial for stem cell function and maintenance are closely associated with the metabolic function of the cell, including HIF-1α, FOXO, or ATM.
Future Directions

Sorting of ROS low and high HSCs linked the levels of ROS to long- or short-term differentiation potential and myeloid differentiation bias. However, ROS was suggested to be not a marker but to have a function in determining the “stem” state of the cell. The function of ROS was shown in HSCs mainly by administrating ROS scavengers, such as NAC, either in vivo or in vitro, and by demonstrating recapitulation of long-term reconstitution potential. NAC inducing reduction of ROS and restoring stem cell ability raised two interesting notions: first, ROS elevation may accompany stem cell switch from dormancy to proliferation, which may also be marked by CD34 expression in murine cells, and second, reduction in ROS levels may render proliferating stem cells into dormancy. Conclusive evidence for these notions is still lacking and the examined populations, including stem cells and progenitors, are too heterogeneous to conclude about a state of a stem cell. It would be exciting to show by analysis on a single cell level that ROS high cells, which expressed CD34 and present a proliferating phenotype, can switch to dormancy and lose their CD34 expression in vivo by ROS level reduction. In this regard, HSCs switch from dormancy to proliferation and mobilization in a circadian manner. It would be interesting to learn whether or not ROS oscillations also exist under the circadian rhythm mediating stem cell proliferation and mobilization.

There seems to be a strict range in which ROS have a beneficial effect on stem cells. ROS levels exceeding this range would lead to senescence of the stem cell and loss of its functionality. Moreover, actual levels of ROS were never determined in the vicinity of the BM, to conclude about the state of the stem cells in the microenvironment in which they reside. A method to directly measure PO2 levels in vivo was recently described (91a). Along with the studies examining morphological locations of hypoxic stem cells (31, 67), these studies will advance our understanding on both cell intrinsic regulation and microenvironmental regulation of the HSC metabolic state. In addition, it would enable monitoring of ROS levels in stem cells, defining the preferential range of ROS levels that promote stem cell cycle without differentiation or entering senescence. This may reveal the possible role of ROS in the different developmental stages of the stem cells, which requires proliferation while preserving the stem cell state.

In addition to their effect in steady state, deregulation of ROS, resulting in their moderately high levels, may also facilitate leukemia. These high levels of ROS not only cause DNA damage or signal for enhanced proliferation, but also reflect an alteration in the metabolic state of the primitive cell. These alterations might have been metabolically rewired to satisfy the increased demands for energy of the fast proliferating cancer cells, while still not producing uncontrolled high levels of ROS that would result in senescence of death of the leukemic blast.

Thus, considering treatment approaches targeted at the altered metabolic pathways found in leukemic stem and progenitor cells might result in new modalities that would be perhaps more leukemic cell specific. The metabolic rewiring of leukemic stem cells still needs to be revealed, but this should follow our understanding of the metabolic profiles of hematopoietic stem and progenitor cells in steady-state hematopoiesis.

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### Abbreviations Used

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>zSMA</td>
<td>z smooth muscle actin</td>
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<tr>
<td>AML</td>
<td>acute myeloid leukemia</td>
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<td>ATM</td>
<td>ataxia telangiectasia mutated</td>
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<td>CAR</td>
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<td>CGD</td>
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