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FORUM REVIEW ARTICLE

Stem Cells, Redox Signaling, and Stem Cell Aging

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Abstract

Significance: Functional stem cell decline has been postulated to result in loss of maintenance of tissue homeostasis leading to organismal decline and diseases of aging. ***Recent Advances:*** Recent findings implicate redox metabolism in the control of stem cell pool and stem cell aging. Although reactive oxygen species (ROS) are better known for their damaging properties to DNA, proteins and lipids, recent findings suggest that ROS may also be an integral physiological mediator of cellular signaling in primary cells. ***Critical Issues:*** Here we review recent published work on major signaling pathways and transcription factors that are regulated by ROS and mediate ROS regulation of stem cell fate. We will specifically focus on how alterations in this regulation may be implicated in disease and particularly in diseases of stem cell aging. In general, based on the work described here we propose a model in which ROS function as stem cell rheostat. ***Future Directions:*** Future work in elucidating how ROS control stem cell cycling, apoptotic machinery, and lineage determination should shed light on mechanisms whereby ROS may control stem cell aging. *Antioxid. Redox Signal*. 20, 1902–1916.

Introduction

STEM CELLS MAINTAIN tissue homeostasis over time by regularly replacing damaged or lost cells. Tissues with high turnover rate, such as blood and skin are maintained by resident stem cells that compensate for the loss of differentiated or damaged cells. Stem cells share two defining properties—pluripotency and self-renewal. Stem cells are pluripotent in that they give rise to all cells within a given tissue (or to all cells within the body). Stem cells also have the capacity to generate with each division, another cell with the stem cell program. This stem cell property is known as self-renewal and enables the organism to maintain a stem cell pool throughout its lifetime. The function of stem cells is known to decline with age, a process that may be implicated in the loss of maintenance of tissue homeostasis leading to organ failure and diseases of aging (Fig. 1) (38).

Adult stem cells are known to be mostly quiescent and to rarely enter cell cycle (38). The few exceptions, such as intestinal stem cells (97), are the subject of debate (72, 87). It has been postulated that quiescence protects stem cells from incurring damage during cell division and plays a necessary role in their lifetime maintenance. Nonetheless, when faced with major tissue loss or damage, stem cells exit their quiescent state and enter the cell cycle to proliferate and generate large numbers of differentiated progenies. These properties may be shared by cancer stem cells (91). Efforts aimed at elucidating the source of the potential of stem cells to regenerate in response to damaged or lost tissues has been the focus of much research in the past over 50 years. Although such studies have reached new heights within the past decade, the basis for the ability of deeply quiescent cells to proliferate on demand is not known. Several recent publications point to metabolism as a potential source for resolving this enigma (35, 110, 129).

Reactive Oxygen Species as Stem Cell Rheostat

Cellular respiration, a process necessary for the survival of all aerobic organisms, involves the oxygen dependent process of oxidative phosphorylation to produce adenosine triphosphate (ATP). The energy released from the hydrolysis of ATP drives essential chemical reactions within the cell. While some ATP production can occur through the anaerobic process of glycolysis, most ATP is synthesized in the mitochondria through oxidative phosphorylation; however, the production

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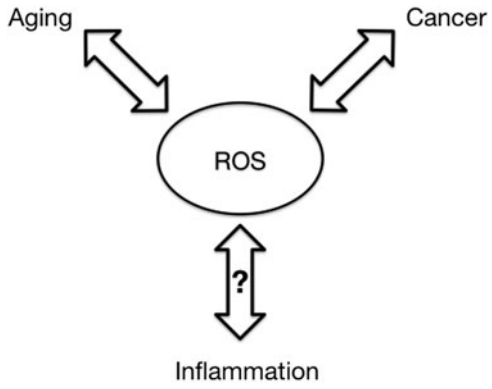

FIG. 1. Contribution of ROS to disease in stem cells. Oxidative stress and aberrant ROS signaling are associated with aging-related diseases including cancer. In turn, these diseases can lead to further dysregulation of ROS signaling and increased oxidative stress. ROS damage is also known to contribute to inflammation; however, the cross-talk between ROS and the inflammatory response in stem cells is unclear.

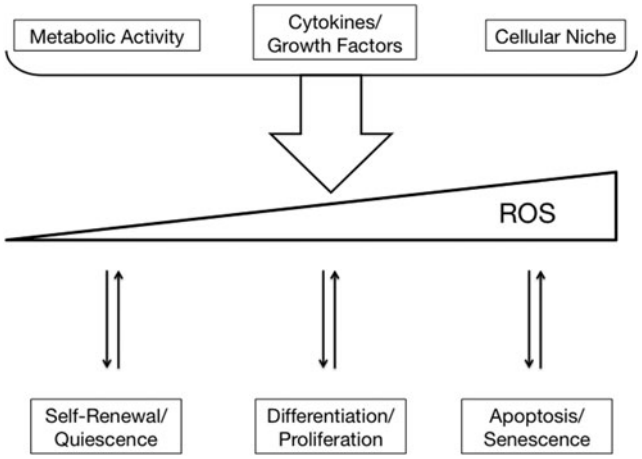

FIG. 2. ROS as stem cell rheostat. Various stem cell intrinsic and extrinsic factors can alter the cellular redox state through the generation of ROS. The cell can sense a gradient of ROS concentrations with multiple proteins in various pathways and respond accordingly. ROS, reactive oxygen species.

of ATP within the mitochondria can lead to the release of harmful reactive oxygen species (ROS) as a byproduct. ROS can adversely react with DNA, lipids, and proteins and the cumulative effects of this damage are thought to contribute to the physiological effects of aging (82). On the other hand, ROS are also considered to be essential components in multiple biological processes as second messengers (21). Emerging evidence increasingly suggests that ROS may not only be a byproduct of metabolic oxidative phosphorylation, but intimately implicated in the physiological regulation of signaling pathways. Alterations in the redox milieu, that is, ROS production versus scavenging, may lead to disease as a result of too much ROS damage, but also perhaps, by means of redox-mediated enhancement (or inhibition) of an otherwise physiologically ROS-regulated signaling protein/pathway (Fig. 1).

ROS are being increasingly implicated in the physiological regulation of critical developmental processes, including the emergence of embryonic blood stem cells (33, 35, 89). A potential model may be that ROS function as a stem cell rheostat by sensing and translating environmental cues into a cellular response to balance cellular output (function) with cellular input (*e.g.*, nutrients, cytokines). Stem cells, in particular, may take advantage of redox regulation to coordinate cell cycle with differentiation as a means of holding their stem cell fate in check, while ensuring homeostasis. Understanding the possible mechanisms by which ROS influence stem cells' fate may provide insights into how the aging of stem cells may be implicated in diseases of aging, as slight variations in ROS content may have profound effects on stem cell fate (Fig. 2).

Source of ROS in Stem Cells

Mitochondria produce almost 90% of ROS in all cells (3). The function of mitochondria in stem cells is an area of active investigation; however, it remains to be determined whether mitochondria are the sole or the major source of ROS in stem cells (Fig. 3). The NADPH Nox family of oxidases is another major source of ROS (8). The primary function of the NADPH Nox family of oxidases is the production of ROS (8). NADPH oxidases are found in phagocytic and nonphagocytic cells, and preferentially use NADPH rather than NADH as substrate. Nox2 and 4 are expressed in human $CD34^+$ hematopoietic stem cells (HSCs) and progenitors (85). The contribution of Nox signaling to the biology of stem cells remains to be seen. It is, however, likely that Nox proteins are involved in the regulation of ROS in stem cells (22, 84, 85). Importantly, the relative contribution of different sources of ROS to the maintenance of tissue homeostasis remains unclear.

ROS in the Regulation of Signaling Pathways

ROS are generated as a consequence of activity by oncoproteins and several cytokine and growth factor stimuli [(45, 46, 74, 98, 106, 111, 117, 134) reviewed in Thannickal and Fanburg (113)]. While the exact mechanism by which cytokine receptor signaling generates ROS in nonphagocytic cells is not fully understood, it is proposed that ROS modulate protein function by oxidization of reactive cysteine residues in signaling proteins [reviewed in Pervaiz *et al.* (82) and Thannickal and Fanburg (113)]. Stimulation of growth factor receptors, such as epidermal growth factor, platelet-derived growth factor (PDGF), and insulin signaling is associated with a transient burst of ROS production that ultimately participates in cellular signaling. This is partly achieved by transient phosphorylation and inhibition of local detoxification enzymes, such as peroxiredoxin I (122). Transient accumulation of ROS around locally concentrated membrane signaling proteins enables ROS to function as signaling messengers (122). ROS impact cellular signaling through protein modifications, such as intramolecular disulfide bridges, sulfonylamide bond formation, direct activation of tyrosine kinases by Cys oxidation, or by inhibition of phosphatases [reviewed in Thannickal and Fanburg (113)]. In turn, the catalytic activity of antioxidant enzymes, such as peroxiredoxins, catalase, and glutathione peroxidase is modified by signaling molecules [reviewed in Rhee *et al.* (92)] suggesting a dynamic balance between cellular signaling and regulation of oxidative stress.

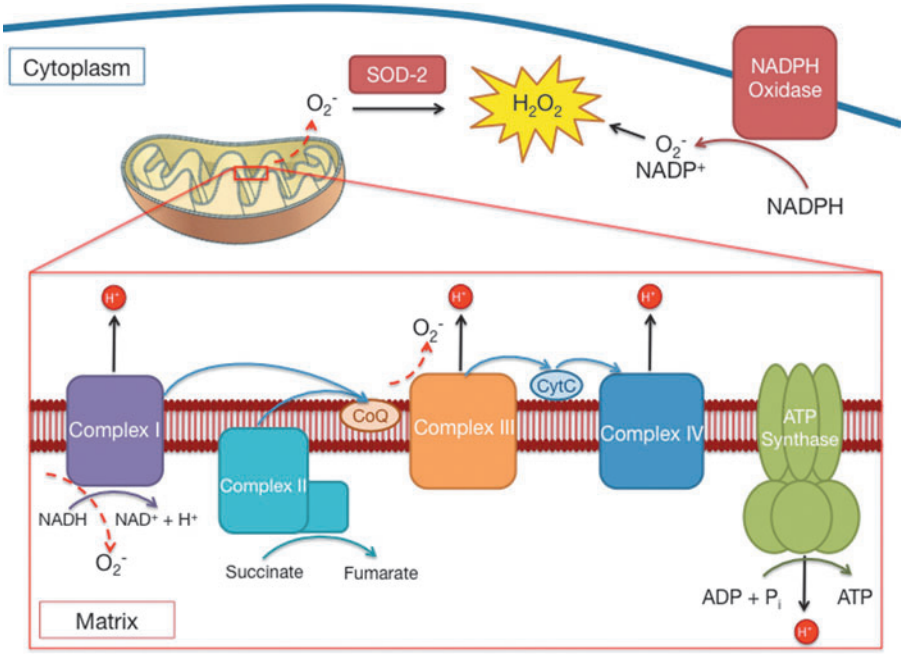

FIG. 3. Sources of ROS. A majority of ROS is produced by the mitochondria mainly through the aberrant reduction of O_2 at complexes I and III during oxidative phosphorylation. The highly reactive and unstable superoxide anion (O_2^-) is scavenged by SOD-1 to form H_2O_2, also a type of ROS. H_2O_2 is more stable and is responsible for direct protein modifications. Another source of intracellular ROS is NADPH oxidase, which produces O_2^- through the oxidation of NADPH. SOD, superoxide dehydrogenase; H_2O_2, hydrogen peroxide. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Many protein tyrosine phosphatases are reversibly oxidized and inactivated by ROS providing a mechanism for ROS-induced mitogenesis (5, 6, 39, 69). In particular, ROS are known to inhibit the function of phosphatase and tensin homology (PTEN), protein tyrosine phosphatase 1B (PTP1B), and CDC25 (68, 69, 99, 101), and inhibit expression of genes such as the one encoding for the adaptor protein Lnk, a negative regulator of cytokine receptor signaling (126). More recently, ROS have been implicated as mediators of a newly discovered nonapoptotic form of cell death termed ferroptosis, which requires mainly iron (19). Importantly, ROS directly regulate the function of a number of proteins implicated in stress response and metabolism (see Table 1). Recent findings describe a physiological function for ROS in the control of disease and developmental processes (33, 35, 78, 89) further supporting a key function for ROS in cellular signaling *in vivo*.

ROS in the Regulation of Stem and Progenitor Cells

The few number of adult stem cells and the lack of easy access to these cells limits to a large extent biochemical and molecular approaches that are routinely used in studies of somatic cells or cultured lines. Nonetheless, work in the past decade has remarkably improved our understanding of ROS regulation of stem cells. ROS are emerging as critical physiological regulators of stem and progenitor cells (Fig. 4). While HSCs have low levels of ROS as compared to their more mature progenies (49), neural stem cells (NSCs) (58) accumulate high levels of ROS that control their proliferation and self-renewal. When bone marrow regions are divided according to their levels of ROS, all long-term HSCs capable of generating more than 20% chimerism 20 weeks after transplantation into lethally irradiated mice are found within the bone marrow compartment with relatively low levels of ROS (49). Specifically, HSCs with lower ROS levels engraft irradiated recipients better than do HSCs with higher ROS content (49). A higher percentage of cells with low ROS levels were in G0 indicating their low cycling status. These cells express high levels of Notch1, cdkn1a (p21), N-cadherin, and CaR and low levels of p16 and mammalian target of rapamycin (mTOR) as compared to ROS high cells enriched for hematopoietic progenitors. In these studies, the low but detectable levels of p53 in ROS low cells were significantly higher than levels found in ROS high cells. These studies also indicated that *in vitro* treatment with either N-acetylcysteine (NAC), a generator of glutathione in scavenging ROS, or inhibitors of p38 mitogen-activated protein kinase (MAPK), or mTOR all led to full recovery of hematopoietic cell activity in culture. Although these latter experiments were not conducted *in vivo*, they suggest that at least some of the ROS-mediated effects on multipotent progenitors are reversible. They also indicate that a connective network of ROS-p38MAPK and mTOR participate in the control of the hematopoietic stem/progenitor cell pool (Fig. 4). Indeed, ROS regulation of p38MAPK has been implicated in the loss of self-renewal of HSC during serial transplantation—a classic unexplained observation regarding HSCs (48). ROS also regulate mTOR directly by controlling the interaction of mTOR with mTOR regulator raptor (95, 128). In turn, activation of mTOR leads to increased oxidative stress in HSCs and loss of HSC function (13).

In agreement with the notion that endogenous ROS regulate fundamental properties of HSC and are implicated in diseases of HSC, several models of ROS-mediated HSC deficiencies provide an explanation for the physiological function of ROS in the control of HSC. Two prominent models are mice deficient in ataxia telangiectasia mutated (ATM) and mice deficient in FoxO3 (or triple FoxO). ATM serine threonine protein kinase is a critical enzyme in the regulation of stress response to DNA damage, specifically double strand DNA break (7). Loss of function mutations in ATM is associated with ataxia telangiectasia that is an autosomal disorder characterized by neuronal degeneration, immunodeficiencies, genomic instability, predisposition to lymphomas and other malignancies, and extreme sensitivity to ionizing radiation

Table 1. Direct ROS Modification of Key Proteins Regulating Stem Cell Function

Protein	*Residue*	*Modification*	*Experimental system*	*Effect*	*Description*	*Reference*
ATM	C2991	Disulfide Bond	Primary human fibroblasts, AT1-ABR	Activating	Oxidation of CYS-2991 activates dimer form of ATM through covalent linkage of two ATM monomers and changes substrate specificity from that of only DNA damage repair proteins to downstream effectors of oxidative stress regulation. However, many downstream proteins regulated by oxidized ATM overlap with those regulated by phosphorylated ATM (induced by double stranded DNA breaks).	(27, 28)
AKT	C297, C311	Disulfide Bond	H9C9, mouse ventral midbrain	Varied	Oxidation of CYS-297 and CYS-311 causes formation of an intramolecular disulfide bond leading to inactivation of AKT through improved association w/PP2A, modification can be reversed by glutaredoxin.	(56, 73, 102)
FOXO	C477-FoxO4	Disulfide Bond	HEK293T, DLD1, MEF	Varied	Dansen *et al.* demonstrate ROS oxidation of CYS-477 on FoxO4 causes association with CBP/p300 through a disulfide bond and subsequent acetylation leading to differential targeting by FoxO4.	(15)
mTORC1	Unknown	Unknown	HEK293T	Activating	Treatment of mTOR with cysteine oxidizers prevents binding of Raptor and enhances kinase activity of mTOR specifically on S6K1. However, treatment with antimycin A, normally causing increased ROS production by mitochondria, had contrasting effects seen with increased association with Raptor and inhibition of S6K1 phosphorylation. Yoshida *et al.* show cysteine oxidizers regulating Rheb, leading to active mTORC1. (No direct modification of mTOR by H_2O_2)	(95, 128)
p53	C141, Y327	Glutathionylation, Nitration	U87MG, HCT116, MCF7, SAOS-2	Varied	Glutathionylation of CYS-141 induced by H_2O_2, inactivates p53 by preventing its dimerization and DNA binding ability. Nitric oxide-mediated nitration of tyr327 enhances p53 nuclear localization and DNA binding activity by increasing oligomerization of p53.	(119, 125)
PTEN	C124, C71	Disulfide Bond	HELA, U87MG, HEK293T, 3T3	Inhibitory	Oxidation of the catalytic cysteine CYS-124 triggers disulfide bond formation with CYS71 and leads to loss of enzymatic activity.	(59, 62, 101)
SIRT	C67-SIRT1	S-Glutathiolation	HEK293T, BAEC	Indirect Inhibition	Oxidation of CYS-67 by S-nitrosoglutathione (GSNO, a product of nitric oxide and glutathione) prevents SIRT1 activation by resveratrol though leading to nitrosylation and subsequent covalent addition of glutathione. Basal activity levels are unaffected.	(131)

PTEN, phosphatase and tensin homology; mTOR, mammalian target of rapamycin; ATM, ataxia telangiectasia mutated; H_2O_2, hydrogen peroxide.

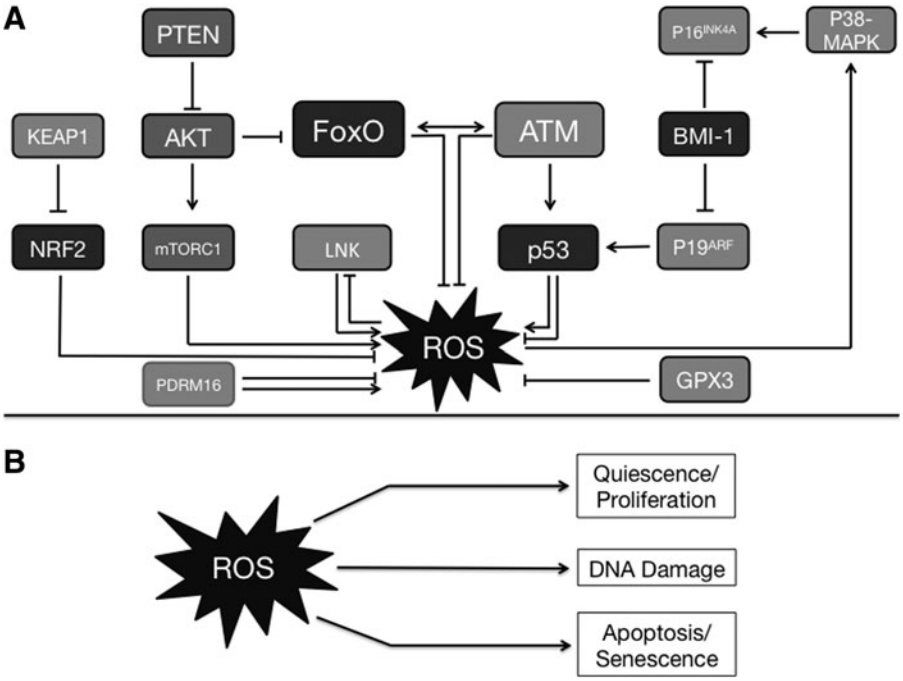

FIG. 4. ROS regulation of signaling pathways in stem cells. (A) In stem cells, a variety of proteins that respond to ROS can determine cellular outcomes, such as whether to enter the cell cycle or remain quiescent. Proteins, including transcription factors p53, PDRM16, FoxO, and NRF2 can, in turn, regulate levels of ROS. **(B)** A schematic of effects of ROS in stem cells. ATM, ataxia telangiectasia mutated

(4). While ROS are found accumulated in several cell types and tissues of ataxia telangiectasic patients (52) and antioxidant enzymes are either upregulated or downregulated in ATM mutant cells, the mechanism of ATM regulation of ROS is not clear (7, 83). ATM-deficient HSC accumulate ROS, and exhibit a ROS-mediated compromise in their maintenance and repopulation ability. ROS accumulation induces upregulation of the cell cycle regulators cyclin-dependent kinase inhibitors $p16^{INK4a}$ and $p19^{ARF}$ in ATM mutant HSC. $p16^{INK4a}$ and $p19^{ARF}$ tumor suppressors (47) are generated from alternative open reading frames of the INK4a locus. $p16^{INK4a}$ and $p19^{ARF}$ inhibit cell cycle progression through inhibition of inhibitors (therefore activation) of retinoblastoma and p53 proteins respectively. Ectopic expression of the polycomb protein Bmi-1, which represses both p16INK4a and p19ARF, restores the defective stem cell function in $ATM^{-/-}$ mice. Subsequent experiments using E6 or E7 human papilloma viral protein expression demonstrated that upregulation of $p16^{INK4a}$ but not $p19^{ARF}$ mediates the HSC defects in ATM knockout mice. Upregulation of $p16^{INK4a}$ in HSCs is an indicator of aging. These studies imply that ROS-mediated upregulation of $p16^{INK4a}$ induces senescence in $ATM^{-/-}$ HSC leading to their functional impairment.

Accumulation of ROS in FoxO- (or FoxO3)-deficient HSC (70, 114, 126), similar to ATM-deficient HSC (47), has been implicated in the loss of HSC quiescence (although, in this case, ROS regulation of FoxO-deficient or FoxO3-deficient HSC self-renewal is yet to be formally demonstrated). FoxO transcription factors exhibit many tumor suppressor functions, such as inhibition of cell cycle, induction of apoptosis and repair of damaged DNA. FoxO have emerged as key regulators of stem cell pluripotency in adult hematopoietic and neural system (FoxO3) (70, 79, 90, 114, 126), as well as in embryonic cells (FoxO1 and FoxO3 in mouse embryonic stem cells (mESC) and FoxO1 in human ESC) (133). In addition, FoxO3 is essential for the maintenance of oocyte pool (11). Interestingly, FoxO (in particular FoxO3) are essential for the maintenance of leukemic stem cells (40, 75, 107). It is not understood how FoxO3 requirement for maintaining leukemic stem cells is compatible with its function as a tumor suppressor.

Loss of FoxO3 alone recapitulates most of the phenotype observed in triple FoxO (FoxO1, −3 and −4)-deleted mice, albeit with less severity (16, 63, 70, 80, 127). Aging exacerbates the defective $FoxO3^{-/-}$ HSC function (70). FoxO3 may control HSC aging via an intrinsic mechanism. It is also conceivable that, by contributing to tissue oxygenation, FoxO3 regulation of red blood cell lifespan (66) may protect HSC from premature aging. FoxO- and FoxO3- deficient HSC exhibit loss of quiescence, while FoxO-deficient HSC are highly cycling, the cycling of FoxO3- deficient HSC is impaired in that they accumulate in the G2/M phase of cell cycle. It is not known exactly how ROS signaling controls HSC quiescence versus cycling; however, $p19^{ARF}$/p53 and $p16^{INK4a}$/Rb have been implicated. As in $ATM^{-/-}$ HSC, $p16^{INK4a}$, that is a critical regulator of HSC activity and senescence (50), was upregulated in $FoxO3^{-/-}$ HSC (127). However, in contrast to $ATM^{-/-}$ HSC, upregulation of $p16^{INK4a}$ in $FoxO3^{-/-}$ HSC was not mediated by ROS. While $p19^{ARF}$ was not significantly modulated in FoxO3 mutant HSC, upregulation of the p53/p21 axis was found to be mediated by the accumulation of ROS (127). In addition, ATM expression is modulated in a ROS-independent manner by FoxO3 in HSC suggesting that FoxO3 is required for ATM transcription (127). In agreement with the notion that FoxO3 and ATM are part of the same network, FoxO3 also regulates the kinase activity of ATM protein (116). These findings together suggest that both ATM and FoxO3 constitute an important node in the regulation of oxidative stress in HSC. Notably, both FoxO and ATM are redox sensors in that their activity is directly regulated by ROS (24, 28).

Similar to the bone marrow niche, ROS levels were used to subdivide the subventricular zone, a NSC containing niche. High ROS brain cells more closely resembled NSCs based on

their ability to form neurospheres in culture. Conversely, cells sorted from the subventricular zone based on known NSC markers exhibit higher levels of ROS compared to nonenriched cells. In these cells, the ROS generating protein NOX is responsible for this increase. It was shown that NOX activation in response to growth factors stimulated the PI3K/AKT pathway through inhibition of PTEN (58). Although, *in vivo* knockdown of NOX or its inhibition significantly decreased proliferation, self-renewal was not affected and more BrdU label retaining cells were present in the subventricular zone. FoxO are also essential for the regulation of NSCs (79, 90). In FoxO3 (as well as in FoxO1, 3, 4) null cells, a redox-mediated gene expression profile has implicated ROS in the loss of NSC function (90). Similar to HSCs, loss of FoxO in NSCs led to loss of quiescence, increased cycling, decreased self-renewal capacity, and also an increase in ROS) (79, 90). Furthermore, treatment with the antioxidant NAC was able to partially rescue the self-renewal defects, but not the increased cycling in FoxO null NSCs (79). Taken together, proliferation of NSCs requires NOX activation and subsequent ROS signaling through the PI3K/AKT pathway, while FoxO may regulate the transition from quiescence to proliferation that leads to either self-renewal or differentiation, partially through reducing ROS levels.

Similar to the regulation of adult stem cells, FoxO proteins regulate the pluripotency of ESC (133). While only FoxO1 (but not FoxO3) is essential for the expression of master regulators of pluripotency, Oct4 and Sox2, in human ESC, FoxO1 and FoxO3 both are implicated in the control of mESC pluripotency. Although it is clear that FoxO1 regulation of pluripotency in human ESC is not mediated by ROS, whether there is any relationship between FoxO and oxidative stress in the regulation of pluripotency in ESC remains unknown.

AKT serine/threonine protein kinases are upstream negative regulators of FoxO that by phosphorylating FoxO promote their cytosolic localization. AKT exists in three isoforms, and their knockout mouse models exhibit distinct phenotypes. Loss of AKT1 or AKT2 alone has only mild effects on HSC (51). Loss of AKT1 and AKT2 together result in increased quiescence of HSC, which remain in G0 and compete poorly against wild-type HSC in reconstitution of lethally irradiated recipients. This is associated with enhanced maintenance of HSC function through serial transplantation and decreased levels of ROS (51). Together, these studies suggest that potential inhibition of FoxO by AKT is functionally important in the control of HSC. Additional work should clarify whether ROS are involved in the regulation of AKT and FoxO3 in HSC.

The p53 tumor suppressor has also been implicated in the control of HSC (65, 112). In contrast to FoxO and ATM, loss of p53 results in increased quiescence of long-term repopulating HSC and is associated with an increased ability to promote competitive repopulation (65, 112) suggesting that maintenance of HSC homeostasis may involve a balance of FoxO, ATM with p53. Interestingly, the increased HSC function seen in p53-null mice is associated with death if the recipient mice are lethally irradiated. Indeed, irradiated mice transplanted with $p53^{-/-}$ HSC succumb to lymphomas within 4 months after transplantation (65, 112).

p53 regulates a number of ROS-inducing genes (p53-induced genes) that modulate apoptosis via control of mitochondria in tumor cells (86). Interestingly, p53, like ATM and FoxO, is also a redox sensor (81). Although p53 has been recently implicated in the control of ROS in postnatal bone marrow (1), it is unclear whether p53 regulation of ROS has any function in postnatal or adult HSC.

The polycomb repressor Bmi-1 is another potential regulator of redox status in HSC. Bmi-1 is essential for the regulation of HSC self-renewal via its control of the Ink4a/Arf locus. Bmi-1 was recently found to be essential for the control of mitochondrial function in thymocytes. Increased ROS levels are associated with a decrease in oxygen consumption and ATP production in thymocytes, and increased ROS levels mediate abnormal thymic function and activation of the DNA damage response in thymocytes. Although ROS levels were also elevated in Bmi-$1^{-/-}$ hematopoietic long-term repopulating cells, the contribution of high ROS levels to the abnormal function of these cells in Bmi-$1^{-/-}$ mice is not known (64). These findings suggest that Bmi-1 (and perhaps other critical regulators of HSC self-renewal) may have important functions in controlling mitochondria and/or controlling the DNA damage response in HSC.

Another redox regulator of stem cells is the PR domain containing 16 (PRDM16) transcription factor (2, 14). PRDM16 is a regulator of human leukemogenesis and brown fat. PRDM16 also regulates redox status in neuronal and HSCs (14). Loss of PRDM16 results in abnormal cycling and significantly increased cell death in stem cells. However, while ROS are increased in PRDM$16^{-/-}$ NSCs and mediate some of the neural stem progenitor defects in PRDM$16^{-/-}$ mice, the levels of ROS in PRDM$16^{-/-}$ HSCs are decreased (14).

In *Drosophila*, ROS are involved in the control of hematopoietic stem/progenitor cell differentiation, as well as homeostasis of intestinal stem cells. Endogenous ROS signaling regulates blood stem cells via FoxO and JNK activation (78). The regulation of intestinal stem cells in *Drosophila* is through the Keap-nuclear factor-erythroid-2-related factor 2 (Nrf2) antioxidant axis (42). It was shown that Nrf2 is constitutively active in ISC. Nrf2 belongs to the cap-n-collar subfamily of the basic leucine zipper (b-Zip) transcription factors and is ubiquitously expressed as a master regulator in the antioxidant response pathway. Loss of Nrf2 in ISCs causes accumulation of ROS and accelerates age-related degeneration of the intestinal epithelium. These findings establish that Keap1 and Nrf2 comprise a critical redox management system that regulates stem cell function, and raise the question as to whether these regulators are involved in other high-turnover tissue stem cells (42). Interestingly, it was recently shown that loss of Nrf2 results in loss of quiescence of HSCs, expansion and hematopoietic stem and progenitors and alterations in cell migration and bone marrow retention suggesting a critical function in the regulation of HSC (115).

Many of the proteins discussed here, such as ATM, p53, FoxO, mTORC1, AKT, and PTEN (15, 24, 27, 28, 56, 59, 62, 73, 95, 101, 102, 119, 125, 128) all of which have important functions in the control of stem cells, are redox sensors, in that their activity is directly modulated by ROS (Table 1). This common property raises the possibility that ROS have a key function in coordinating the interactions of the PTEN/AKT/mTOR/FoxO pathway with ATM and p53. It is also tempting to speculate that mechanisms that control ROS in stem cells are directly implicated in the control of stem cell fate. It remains to be seen whether redox state is implicated in the regulation of these proteins in stem cells.

The gradual loss of function through serial transplantations or in long-term suspension cultures are well recognized features of HSC (36, 37). ROS are recognized as potential mediators of the loss of HSC function in these settings as antioxidant treatment improves (almost normalizes) HSC function throughout serial transplantation and in long-term *in vitro* culture (48). ROS activation of p38-MAPK is implicated in limiting HSC function both *in vivo* and *in vitro* (48). Exactly how ROS control HSC function is not known. One potential mechanism is through the regulation of the response to DNA damage or through regulation of DNA repair. In support of this, there is recently reported evidence that accumulation of oxidative DNA damage restricts the self-renewal capacity of human HSCs (124).

ROS, Aging, and Malignancy

Aging is associated with reduced tissue regeneration leading to increased degenerative disease and cancer. Stem cells regenerate many adult tissues and are implicated in the development of cancer. It is therefore, thought that age-related changes in stem cells may contribute to age-related disease. Consistent with this, stem cell function declines with age in numerous tissues as result of accumulation of DNA damage, mitochondrial damage, epigenetic and environmental modulations, etc. Whether and to what extent ROS contribute to the aging of stem cells is an active area of research. Excessive ROS concentrations lead to abnormal proliferation, growth, and malignancies (47, 77). This has been particularly illustrated in peroxiredoxin 1-deleted mice, which exhibit severe ROS-mediated hemolytic anemias and tumor formation (lymphomas, carcinomas and sarcomas) (77) later in life, and in superoxide dismutase 2-deleted mice, which exhibit oxidative stress mediated hematopoietic abnormalities (23, 67).

Aged HSC are compromised in their regeneration and tissue repair capacity. Aging is associated with HSC expansion, compromise of their homing, gradual loss of their function, HSC myeloid biased-differentiation, and onset of anemia (20, 93, 103). Studies in the past decade suggest redox pathways contribute to the HSC aging. Specifically, alterations in the regulation of ROS by FoxO3 and ATM with age contribute to HSC aging (47, 48, 70). However, underlying mechanisms remain unknown. The importance of the PTEN/AKT/mTOR/FoxO signaling pathway in the control of ROS in stem cells, as well as its involvement in the control of cellular aging, raises the possibility that the regulation of ROS may be implicated in the rate of stem cell aging.

Myeloproliferative neoplasms (MPN) encompass a number of preleukemic blood stem cell disorders that occur with increasing frequency with age and result in the expansion of a myeloid lineage. FoxO-triple mutant and FoxO3$^{-/-}$ mice exhibit myeloproliferative activity reminiscent of human MPN (114, 126). The myeloproliferation in FoxO3$^{-/-}$ mice is characterized by over accumulation of ROS, which are the principal mediator of amplification of the FoxO3$^{-/-}$ hematopoietic progenitor pool and of myeloproliferative-like syndrome in mice (126). ROS activation of the AKT/mTOR signaling pathway as part of a feedback loop has been implicated in the myeloproliferation. Consistent with these findings, constitutive activation of AKT (a negative regulator of FoxO) in mouse HSCs leads to a myeloproliferative disease associated with lymphoblastic thymic T cell lymphoma. A small but significant percentage of these mice develop acute myeloid leukemias (AML) without pre-existing MPN, although AML is not seen in FoxO3$^{-/-}$ or triple FoxO-deleted mice. The AKT-driven disorders in this model are not redox sensitive, but treatment with rapamycin to inhibit mTOR signaling was found to partially improve the T cell lymphoma (54). In contrast, the MPN (or AML) in this model (in contrast to MPN in FoxO3$^{-/-}$ mice) was not sensitive to rapamycin. Nonetheless, in other models, AML seems to be sensitive to rapamycin (132).

The analysis of myeloproliferation in FoxO3$^{-/-}$ mice also suggests that ROS-mediated regulation of SH2B3 (Lnk) adaptor protein expression significantly enhances the sensitivity of cytokine signaling to changes in physiological ROS, and leads to increased cycling and expansion of FoxO3 mutant hematopoietic progenitor cells. Lnk is a negative regulator of cytokine receptor signaling (108, 118) that binds to JAK2 protein tyrosine kinase (and thrombopoietin receptor) and downregulates thrombopoietin receptor signals that are essential for supporting HSC. In *Drosophila*, dFoxO represses Lnk expression directly. Also, in *Drosophila*, Lnk negatively regulates lifespan via control of oxidative stress (105). Overall, Lnk is a negative regulator of HSC (10). Lnk$^{-/-}$ HSC are increasingly quiescent and exhibit enhanced self-renewal (10). Loss of Lnk may contribute to protecting HSC from aging (9). Consistent with the model of ROS as a stem cell rheostat, the levels of ROS are lower in Lnk$^{-/-}$ HSC than in wild type controls (Bigarella, Liang and Ghaffari, unpublished findings). These findings raise the possibility that modulations of ROS are highly connected to HSC function and aging, and to longevity of the whole organism, the point where the stem cell theory of aging and the oxygen radical theory of aging may intersect.

Sirtuins, a family of NAD-dependent deacetylases (with seven members in mammals), are implicated in the regulation of aging, oxidative stress, and metabolism. SIRT1 controls apoptosis of mouse embryonic stem cells (mESC) by regulating nuclear *versus* mitochondrial localization of p53 in response to endogenous oxidative stress (34) (Fig. 5). SIRT1 is further implicated in the regulation of mitochondrial biogenesis in skeletal muscle (88). Whether SIRT1 has any function in the redox regulation of adult stem cells remains unknown (131). Interestingly, recent findings suggest that SIRT3, another member of the sirtuin family, which has a critical function in the maintenance of mitochondria and metabolism (55), is essential for the control of ROS during aging of HSC. SIRT3 was shown to enhance the function of old HSC, suggesting that aging of stem cells may be reversible. As old HSC are implicated in blood malignancies, such as leukemias, MPNs, and myelodysplasia, the possibility that SIRT3 plays a role in the initiation or maintenance of these disorders requires further investigations. Similarly, it will be interesting to determine if polymorphism in SIRT3 expression (or modulation of SIRT3 deacetylase activity) is associated with longevity, as has been proposed for FoxO3 (120, 121).

HSC and Mitochondria

Studies of liver kinase B1 (LKB1) knockout mice have revealed important functions for mitochondria in stem cells (94). Loss of LKB1 (25, 29, 76), an evolutionarily conserved

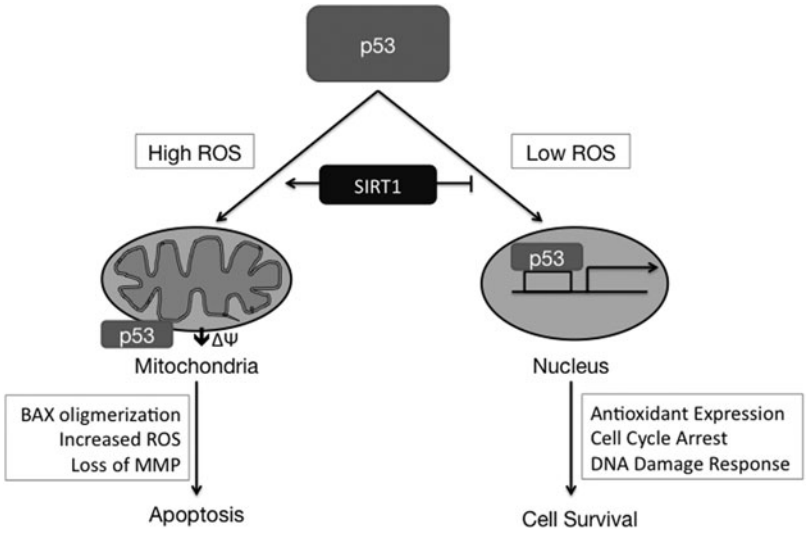

FIG. 5. SIRT1 mediated apoptosis in mESCs [adapted from Gurumurthy *et al.* (29)]. In response to ROS, p53 can either induce apoptosis, under high ROS concentrations, or initiate events that promote cell survival, under low ROS conditions. Deacetylation of p53 by SIRT1 prevents nuclear translocation and promotes the proapoptotic response elicited by cytosolic p53. mESC, mouse embryonic stem cells.

regulator of cellular energy metabolism, leads to pancytopenia, loss of HSC reserves, decreased mitochondrial biogenesis, reduced energy production, reduced mitochondrial membrane potential, and reduced ATP levels. LKB1 is an upstream kinase acting on adenine monophosphate-activated protein kinase (AMPK) (Fig. 6). By phosphorylating tuberous sclerosis complex, AMPK negatively regulates mTORC1. Although loss of LKB1 leads to the activation of mTORC1, ROS levels are not altered in $LKB1^{-/-}$ or $AMPK^{-/-}$ HSC (or any other hematopoietic cell examined), and the LKB1 HSC phenotype seems to be mTOR-independent. Loss of AMPK function leads to mitochondrial defects and mildly altered HSC function and decreased long-term multilineage reconstitution but does not phenocopy the effects of LKB1. A critical outcome of these studies is that the regulation and function of LKB1/AMPK/mTORC1 seems to be specific to HSC and distinct from their function in more committed hematopoietic progenitors. Loss of LKB1 leads to defective centrosomes and mitotic spindles with consequent aneuploidy. These studies illustrate the complex and specific function of metabolic pathways and mitochondria in the control of HSC cycling and dormancy; the results suggest that tight regulation of energy metabolism is critically involved in the control of mitosis in stem cells.

Signaling by mTOR protein kinase has also been implicated in the control of mitochondria. mTOR is a critical regulator of normal hematopoietic and leukemic stem cells. Loss of Tsc1, an upstream inhibitor of mTOR, leads to mTORC1 activation, loss of HSC quiescence, enhanced mitochondrial biogenesis, and ROS accumulation leading to HSC activation and progressive loss of the HSC pool (13). Loss of HSC activity is partially restored by scavenging ROS via administration of NAC *in vivo*. As mTOR signaling is increasingly implicated in the regulation of longevity, it will be interesting to determine if mTOR regulation of stem cells and aging are interrelated.

HIF-1 Mediated Hypoxic Response

The hypoxic response occurs at cellular O2 levels ranging from 0.3% to 3%, and is regulated by the master transcriptional regulator of hypoxic response (hypoxia-inducible factor [HIF]). To maintain oxygen homeostasis at normoxic levels, the response to hypoxia is two-fold. First, the cell decreases oxygen consumption by inhibiting ATP intensive processes and switching to glycolytic metabolism. Second, the tissue increases the local oxygen supply through angiogenesis and erythropoiesis (60). The hypoxic response is critical for stem cell maintenance (61, 104). The HIF-1 complex, a heterodimer of a HIF-α subunit and a HIF-1β subunit, activates most of the genes that induce angiogenesis, erythropoiesis, and glycolysis, including vascular endothelial growth factor, erythropoietin, and various glycolytic enzymes. HIF-α has three

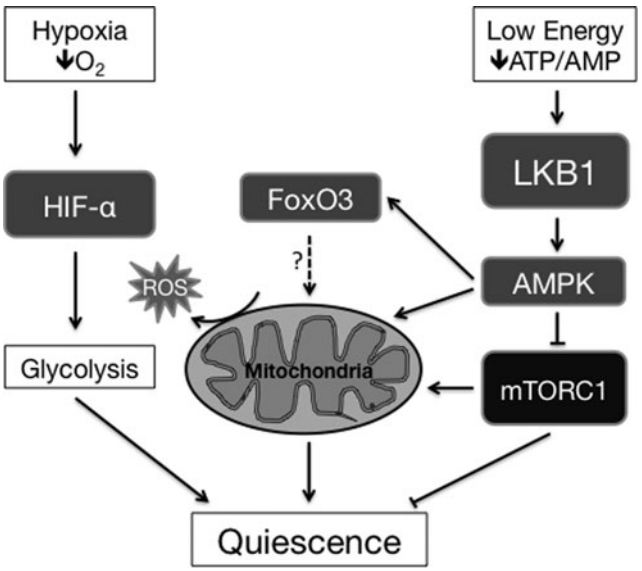

FIG. 6. Mitochondrial and metabolic control of HSC quiescence. While glycolytic metabolism is crucial for maintenance of HSCs in quiescence, functional mitochondria are also necessary. LKB1 and AMPK can be activated upon low energy conditions to regulate mitochondrial function and biogenesis, partially through the inhibition of anabolic processes mediated by mTORC1. FoxO3 may also have a potential role in regulating mitochondrial function. HSC, hematopoietic stem cells; AMPK, adenine monophosphate-activated protein kinase; LKB1, liver kinase B1.

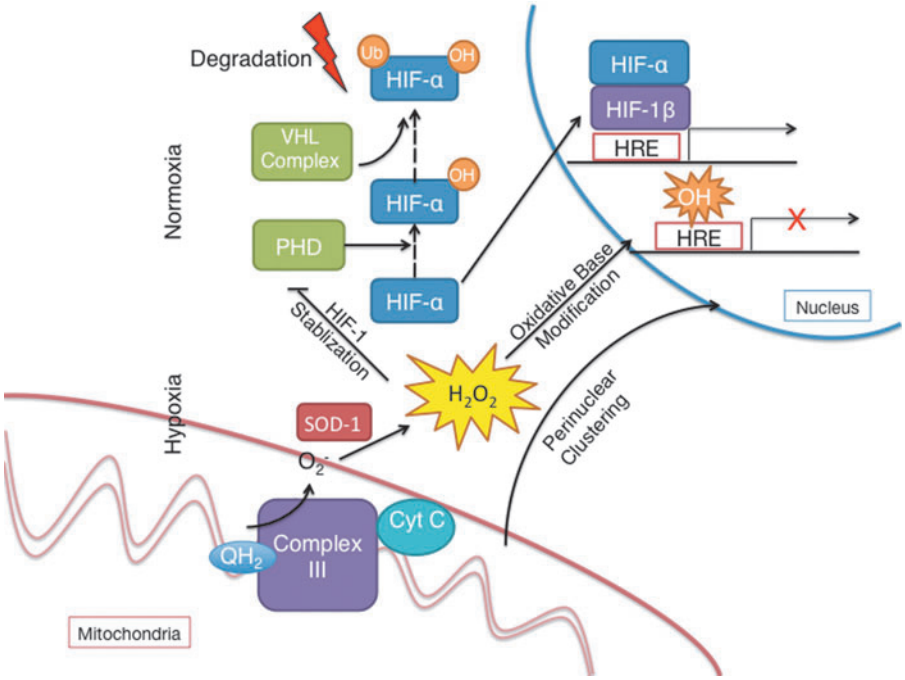

FIG. 7. ROS mediated HIF response. Under normoxia, HIF-1α and HIF-2α are hydoxylated by prolyl hydroxylases, allowing them to be recognized by the VHL complex. The VHL complex polyubiquinates HIF-α leads to proteosomal degradation. Under hypoxia, mitochondrial complex III increases generation of cytosolic superoxide, which is quickly dismutated into hydrogen peroxide. The rise in hydrogen peroxide activates HIF-α by preventing PHD mediated HIF-α hydroxylation. Furthermore, it elicits perinuclear clustering of mitochondria and oxidative base modifications that are associated with transcriptional activation at HREs. HIF, hypoxia-inducible factor; HRE, hypoxic response elements. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

isoforms, HIF-1α, HIF-2α, and HIF-3α, which exhibit differing tissue specificities and regulate different genes. Both HIF-α and HIF-β subunits are expressed constitutively, but HIF-α stability is dependent on oxygen availability. Under normoxia, HIF-α is hydroxylated at proline residues within its oxygen dependent domain by prolyl hydroxylases (PHD) (Fig. 7). PHD function is dependent on oxygen as a substrate and requires the cofactors α-ketoglutarate and iron (100). After hydroxylation, HIF-α becomes a target of the Von Hippel-Lindau protein (pVHL) E3 ubiquitin ligase. Ubiquitination of HIF-α allows for recognition and subsequent proteasomal degradation (53). Under hypoxia, PHD loses the ability to modify HIF-α leading to its stabilization and heterodimerization with HIF-1β.

Unexpectedly, multiple *in vitro* studies have shown that an increase in ROS production stimulates the hypoxic response to low cellular oxygen (1.5% O_2) (12, 32). Details of the pathway have yet to be elucidated, but complex III of the mitochondrial electron transport chain is a probable source of the enhanced generation of ROS that have been linked to stabilization of HIF1a. There are also several reports that hypoxia-generated ROS oxidize specific residues in hypoxia response elements (HREs) of hypoxia-inducible genes. On the basis of multiple lines of indirect evidence, it has been speculated that hypoxia-induced base modifications might contribute to transcriptional regulation (see Fig. 7). However, isolated mitochondria actually produce lower amounts of ROS as concentrations of oxygen decrease. This suggests that a decrease in oxygen concentration alone is not sufficient to increase ROS production, and upstream factors that increase superoxide generation by complex III are necessary. Importantly, hypoxia-induced ROS are required for activation of the hypoxic response (12, 130). The contradictory roles of mitochondrial ROS raise the question as to whether ROS generation results from inefficiencies within the electron transport chain or is a tightly regulated process. The debate as to whether mitochondrial ROS are required for HIF signaling is specifically of interest to stem cell biologists, as localization of adult stem cells in hypoxic niches is critical to the maintenance of the stem cell pool, and HIF1α is critical for the maintenance of stem cells (109). Although ROS levels measured by Mito-Tracker Orange (CM-H_2TMRos) are increased in HIF-1α-deleted HSC, the potential function of ROS in this context in stem cells has not been evaluated (109).

Cancer Stem Cells

The cancer stem cell hypothesis postulates that most tumors are highly heterogeneous in their cellular components and only a small subset of cells within the tumor that have self-renewing potential maintain the disease. Although the molecular bases of cancer stem cell self-renewal are unknown, it is hypothesized that many cancer stem cells share many of the same regulatory mechanisms as normal adult stem cells (91). The levels of ROS prove to be of importance in cancer stem cells, the self-renewing and tumorigenic subpopulation within a tumor. Cancer stem cells have been functionally characterized in multiple types of cancers through their ability to generate a tumor after transplantation into a new animal and can be distinguished by their cell surface markers. Diehn and Majeti show ROS levels are significantly lower in CSCs derived from murine and human solid tumors when compared to nontumorigenic cells from matched tumor masses when measured by dichlorofluorescein diacetate (DCF-DA), a marker of H_2O_2 (17, 18). However, when murine CSCs are sorted according to ROS concentration based on DCF-DA intensity, both low and high ROS populations are able to establish tumors in recipient mice, which suggest ROS levels alone do not determine tumorigenicity. Nevertheless, the lower levels of ROS in cancer stem cells are attributed to an upregulation of antioxidant genes that functionally results in increased resistance to free radical damage induced by

ionizing radiation. DNA damage assays demonstrate that CSCs incur less DNA damage compared to the nontumorigenic cells after irradiation. Furthermore, inhibition of ROS scavenging by CSCs sensitizes them to irradiation, and conversely, addition of antioxidants to nontumorigenic tumor cells provides protection against irradiation.

Like adult stem cells, cancer stem cells exhibit low accumulation of ROS as compared to their normal tissue counterparts. They are exquisitely sensitive to ROS, equipped with a strong antioxidant and DNA damage repair arsenal and are highly sensitive to antioxidant depletion (17, 18). Evidence from studies of parthenolide, a naturally occurring molecule, in human AMLs suggests that ROS production preferentially targets leukemic cells and induces apoptosis (30, 31). The set of genes enriched for in CSC's mainly involve glutathione metabolism, a protein required for H_2O_2 scavenging. A similar phenomenon is seen in leukemic stem cells (LSC), which overexpress glutathione peroxidase 3 (GPx3) relative to normal leukemic cells (41). GPx3 is an enzyme that catalyzes the oxidation of glutathione by H_2O_2 to prevent it from forming more toxic intermediates. The high levels of GPx3 lead to low levels of ROS within LSCs and provide them with a greater proliferative capacity shown by comparison with GPx3 knock down LSCs in a competitive repopulation assay. Additionally, mRNA levels of GPx3 correlate well with clinical severity of AML. In summary, CSCs gain various methods of scavenging ROS through increased expression of antioxidant genes, which in turn increase their self-renewal potential and protect against irradiation. These findings indicate that interfering with ROS management may provide a potential target for therapy of cancer. More recently, low levels of ROS have been associated with leukemic stem cell properties of human AML providing an opportunity to target these cells directly (57). Directly comparing the high ROS to the low ROS AML cell population, instead of segregating leukemic stem cells based on established cell surface markers, revealed that the low ROS fraction possess a lower metabolic rate dependent on oxidative phosphorylation. Disruption of mitochondrial energy production by targeting BCL-2, a gene found highly upregulated in the low ROS population, selectively induced an increase in ROS and apoptosis in low ROS cells (57). Intriguingly, addition of antioxidants could not rescue BCL-2 inhibited low ROS containing AML cells, suggesting the mechanism of killing is not through increased ROS. Whether directly targeting the antioxidant machinery of the low ROS fraction could also eradicate them, was untested. Nevertheless, cancer stem cells appear to contain low levels of ROS that are associated with functional benefits, such as radioresistance and self-renewal potential, which makes targeting this population crucial in eliminating the tumor.

Along the same lines, targeting proteins that regulate ROS levels could potentially be an effective way of killing CSCs. Using a histone deacetylase inhibitor (HDACi) against leukemia cell lines demonstrates this effect, seen through the induction of cell death as a result of increased ROS levels (44). HDACi stimulates activity of NADPH oxidase, one of the main sources of cellular ROS, while subsequently causing nuclear translocation of NRF2, a transcription factor controlling many of the genes responsible for antioxidant activity. Furthermore, a synergistic relationship as measured by cell death is observed when cells are treated with a drug that depletes glutathione, β-phenylethyl isothiocyanate after treatment by an HDACi. The same combinatorial treatment can also overcome HDACI resistance, which arises due to the transcriptional upregulation of antioxidant genes by NRF2. The cytotoxicity of the drugs can be inhibited through pretreatment with NAC, a molecule, which enhances glutathione synthesis. Although these experiments do not distinguish between CSCs and the rest of the leukemic cell population, modulating ROS levels proves to be an effective way of targeting leukemia.

ROS may participate in the events that initiate leukemia and be a potential contributor to leukemic transformation. Experimental evidence show ROS promoting growth factor independent proliferation, an initial step of tumorgenesis, in hematopoietic progenitors induced by ectopic expression of constitutively active RAS (43). RAS activation leads to ROS production by NADPH oxidase, which together with RAS increases proliferation of transfected hematopoietic progenitors cultured in serum and growth factor free media. It is important to note that although proliferation increases, survival is diminished in cells transfected with oncogenic RAS as an effect of increased ROS demonstrated by the increase in percent cell viability of transfected cells when treating with antioxidants.

Stem Cells in the Clinic

One of the most promising applications of stem cells is to utilize the self-renewal and multipotent nature of stem cells as a therapy to replenish various cell types and tissues destroyed by disease. However, because stem cells samples are precious and not easy to acquire, storage of samples remains an important objective. Research into the role of ROS in stem cells can lead to more efficient methods of cryopreservation, which allows for the long-term storage of cells so they can be used at later time points for therapeutic or research uses. The technique employs slowly freezing cells to a temperature of $-196°C$ in the presence of dimethylsulfoxide, both elements that reduce the formation of damaging ice crystals (96). Although most mammalian cell types can be cryopreserved and recovered with standard protocols, efficient recovery of cryopreserved stem cells is much more challenging. The reason being that after freezing and thawing there is a significant increase in ROS, which stem cells are uniquely sensitive to. The increase in ROS after cryopreservation was shown to induce apoptosis in human embryonic stem cells and contribute to the poor recovery rate (123). In subsequent studies, incorporating methods that minimize ROS levels over the whole cryopreservation process led to increased viability and cell recovery (71, 96).

Concluding Remarks

Regulation of ROS is clearly of critical importance to adult stem cells. Less is known about ROS regulation of embryonic stem cells. Although key transcription factors and signaling proteins have been identified, the exact mechanism by which ROS control stem cell transcriptional network is unknown. Future studies on how ROS interconnect cell cycle and apoptotic machinery, whether ROS impact the stem cell epigenome as well as whether and how ROS influence stem cell fate determination, stem cell transformation and aging should clarify the details, as well as the importance, of the ROS regulated network in adult and embryonic stem cell biology.

Acknowledgments

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Abbreviations Used

AML = acute myeloid leukemia
AMPK = adenine monophosphate-activated protein kinase
ATM = ataxia telangiectasia mutated
ATP = adenosine triphosphate
DCF-DA = dichlorofluorescein diacetate
ESC = embryonic stem cells
GPx3 = glutathione peroxidase 3
H_2O_2 = hydrogen peroxide
HDACi = histone deacetylase inhibitor
HIF = hypoxia-inducible factor
HRE = hypoxic response elements
HSC = hematopoietic stem cells
LKB1 = liver kinase B1
MAPK = mitogen-activated protein kinase
mESC = mouse embryonic stem cells
MPN = myeloproliferative neoplasms
mTOR = mammalian target of rapamycin
NAC = N-acetylcysteine
Nrf2 = nuclear factor-erythroid-2-related factor 2
NSC = neural-stem cell
PRDM16 = PR domain containing 16
PTEN = phosphatase and tensin homology
ROS = reactive oxygen species
SOD = superoxide dehydrogenase