Reactive Oxygen Species in Cancer Stem Cells

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Abstract

Significance: Reactive oxygen species (ROS), byproducts of aerobic metabolism, are increased in many types of cancer cells. Increased endogenous ROS lead to adaptive changes and may play pivotal roles in tumorigenesis, metastasis, and resistance to radiation and chemotherapy. In contrast, the ROS generated by xenobiotics disturb the redox balance and may selectively kill cancer cells but spare normal cells. *Recent Advances:* Cancer stem cells (CSCs) are integral parts of pathophysiological mechanisms of tumor progression, metastasis, and chemo/radio resistance. Currently, intracellular ROS in CSCs is an active field of research. *Critical Issues:* Normal stem cells such as hematopoietic stem cells reside in niches characterized by hypoxia and low ROS, both of which are critical for maintaining the potential for self-renewal and stemness. However, the roles of ROS in CSCs remain poorly understood. *Future Directions:* Based on the regulation of ROS levels in normal stem cells and CSCs, future research may evaluate the potential therapeutic application of ROS elevation by exogenous xenobiotics to eliminate CSCs. *Antioxid. Redox Signal.* 16, 1215–1228.

Introduction

REACTIVE OXYGEN SPECIES (ROS), including superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl free radical ($HO^\bullet$), are formed by the capture of electrons by an oxygen atom (43). Low-to-moderate levels of ROS are essential for cellular proliferation, differentiation, and survival (113). Normal cells regulate the intracellular ROS content within a nontoxic range by balancing the ROS generation and scavenging systems. Chronically increased endogenous ROS lead to adaptive changes that play pivotal roles in tumorigenesis, metastasis, and drug resistance in diverse types of cancer cells. However, ROS generation by xenobiotics may disturb the redox balance and selectively kill cancer cells without significant toxicity to normal cells (113).

Cancer cells are believed to be derived hierarchically from a small subset of malignant cells that have a high capacity of self-renewal and differentiation—namely cancer stem cells (CSCs) or tumor-initiating cells (84). CSCs have the high potential to generate tumors. For instance, CSCs in human acute myeloid leukemia (AML) harbor $CD34^+/CD38^-$ surface antigens and have the potential to induce leukemia when transplanted into nonobese diabetic severe combined immunodeficiency disease (NOD/SCID) mice, while non-$CD34^+/CD38^-$ cells do not have this potential (102). In a xenograft model of human breast cancer, as few as 200 CSCs harboring $CD44^+/CD24^-$ surface antigens were able to form tumors in immunocompromised mice, while more than 500,000 non-$CD44^+/CD24^-$ cells were not able to do so (6, 107). Similarly, 100 brain tumor CSCs identified as $CD133^+$ cells could form tumors with the same phenotype as the original tumor when transplanted into NOD/SCID mouse brains, while 100,000 $CD133^-$ tumor cells could not do so (105). These findings support the characterization that CSCs have a high potential to grow into tumors.

The biological effects of ROS and the biological mechanisms regulating the level of ROS have been studied in cancer cells as a whole, and little is known about these issues specifically in the subpopulation of CSCs. Although evidence has accumulated in normal stem cells, particularly in hematopoietic stem cells (HSCs), a significant gap in knowledge remains in malignant stem cells. This review will focus on the discussion on the regulation of ROS levels in normal stem cells and CSCs, and the potential therapeutic implication of ROS elevation by exogenous xenobiotics to eliminate CSCs.

ROS and Their Regulatory System

ROS are broadly defined as oxygen-containing reactive chemical species. ROS are mainly divided into two classes: free radical ROS and nonradical ROS. The former class, including O_2^-, nitric oxide ($NO^\bullet$), and $HO^\bullet$, is characterized by containing one or more unpaired electron(s) in their outer molecular orbital. The latter class includes H_2O_2, ozone, peroxynitrite ($ONOO^-$), and hydroxide, which do not have unpaired electron(s) but are chemically reactive to generate

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free-radical ROS under certain conditions with or without enzymatic catalysis (Fig. 1) (113). Under physiologic conditions, low-to-moderate levels of ROS interact with macromolecules by reversible oxidative modifications. This process is crucial for cell development because of the wide involvement of ROS in cellular proliferation, differentiation, and trafficking of intracellular vesicles (94, 113). Additionally, ROS elevation is an important mechanism that eliminates pathogens and foreign particles in phagocytic cells (120). However, due to their high chemical reactivity, excessive amounts of ROS, regardless of endogenous or exogenous origin, can lead to cellular senescence, death, or transformation by causing the irreversible peroxidation of lipids, amino acids, nucleic acids, and carbohydrates (94, 114).

Oxidative stress damages both nuclear DNA and mitochondrial DNA and initiates DNA repair signaling pathways (4, 45). Lipid peroxidation is caused by $HO^{\bullet}$. Lipid oxidation and lipid peroxidation products (*e.g.*, malondialdehyde, 4-hydroxy-2-nonenal) impair biological membrane systems (86). Oxidation in the Fe-S cluster of many proteins may elicit disulfide bond-mediated protein cross-linkage and adduct formation, disabling proteins such as enzymes involved in the mitochondria electron transport chain (ETC). In the case of oxidative modification of 26S and 20S proteasomes, cells may die because of insufficient elimination of damaged proteins (121).

Generation of ROS

The principal source of ROS in mammalian cells comes from the byproducts of electron leakage of mitochondrial ETC. The mutations in nuclear or mitochondrial genes en-coding the ETC components can block the electron transfer, leading to electron leakage (Fig. 1). The electrons can then be captured by O_2, forming O_2^-, which is usually converted to H_2O_2 by manganese (Mn)-containing mitochondrial superoxide dismutase (MnSOD, SOD_2), Cu/Zn-containing cytosolic SOD_1 or extracellular SOD_3 (89). H_2O_2 diffuses to the nucleus to attack chromosomal DNA. H_2O_2 can be catalyzed to release $HO^{\bullet}$ in the presence of Fe^{2+} or Cu^{2+} ions, which is the Fenton reaction (113). In macrophages and cancer cells, O_2^- may additionally be generated by a reaction catalyzed by the membrane-located NAD(P)H oxidase complex (NOX) consisting of NOX1, NOX2, NOX3, NOX4, cytochrome c oxidase, cyclo-oxygenase, and endoplasmic reticulum-associated xanthine oxidase (XO) (Fig. 1). $NO^{\bullet}$ is generated from arginine catalyzed by nitric oxide synthase (NOS), which has a diverse tissue distribution with some tissue-specific isoforms: mitochondrial NOS, neuronal NOS, endothelial NOS, and inducible NOS (34). $NO^{\bullet}$ is rapidly converted to $ONOO^-$ by reacting with O_2^-. $NO^{\bullet}$ and $ONOO^-$ are also referred to as reactive nitrogen species (RNS). Being oxidative, RNS are often included as ROS. $ONOO^-$ can cause the nitration of tyrosine residues, which impairs the activity of the proteins by blocking the phosphorylation and adenylation of these tyrosine residues (22).

Elimination of ROS

The intracellular ROS are maintained at low levels in mammalian cells by powerful scavenger antioxidative enzyme systems, including SOD_1, SOD_2 and SOD_3, catalase, glutathione peroxidase (GPX), and peroxiredoxin. As mentioned earlier, O_2^-, once formed, is rapidly converted by
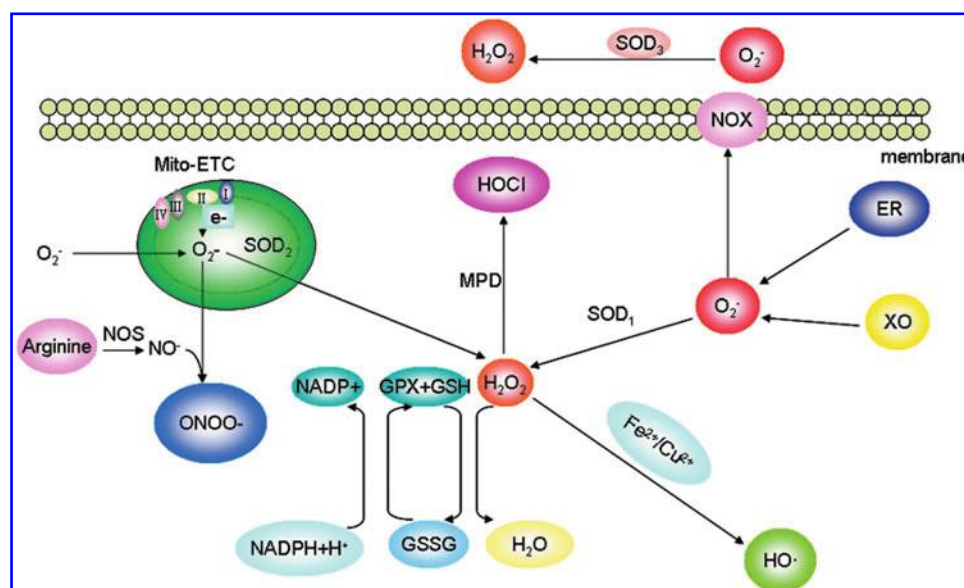

FIG. 1. Cells maintain redox homeostasis through a balance of generation and elimination of reactive oxygen species (ROS). Superoxide (O_2^-), the principal form of ROS, comes from the byproducts of electron leakage of mitochondrial electron transport chain (Mito-ETC), endoplasmic reticulum (ER), and membrane-located NAD(P)H oxidase complex (NOX). O_2^- can be rapidly converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD); H_2O_2 can be catalyzed to release hydroxyl radicals ($HO^{\bullet}$) in the presence of Fe^{2+} or Cu^{2+} ions. H_2O_2 can be converted by myeloperoxidase (MPD) to hypochlorous acid (HOCl), a stronger oxidant. H_2O_2 is converted to $H_2O + O2$ by catalase or glutathione peroxidase (GPX). Nitric oxide ($NO^{\bullet}$), once generated from arginine catalyzed by nitric oxide synthase (NOS), is rapidly converted to peroxynitrite ($ONOO^-$) by reacting with O_2^-. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

SODs to H_2O_2 (Fig. 1). H_2O_2 is converted to H_2O+O_2 by peroxisome-located catalase. H_2O_2 is also converted by GPX to H_2O+O_2 through coupling with the conversion of reduced glutathione (GSH) to oxidized glutathione (113). The conversion of H_2O_2 to the highly reactive $HO^{\bullet}$ is catalyzed by Fe^{2+}/Cu^{2+} (the Fenton reaction) (113). In macrophages, H_2O_2 can be converted by myeloperoxidase to hypochlorous acid, a strong bacteria-killing oxidant (120).

ROS and Stem Cells

Concept of stem cells

Stem cells are characterized by a high capacity of self-renewal and differentiation (2). Through self-renewal, stem cells maintain the homeostasis of a stem cell pool; through differentiation, stem cells differentiate into terminal cells with diverse morphology and functions. Stem cells are present in at least several types of tissues such as the bone marrow, digestive tract, central nervous system, and epidermis and play an important role in tissue repair and homeostasis (2, 96). Most stem cells in tissues such as bone marrow are in the quiescent state (G_0 phase), and they are protected by special microenvironments (niches) (83). The quiescence of stem cells may protect them from the accumulation of DNA replication errors (8). Relatively more cycling stem cells are present in fast-turnover tissues such as intestinal epithelium, mammary epithelium, and hair follicles to maintain homeostasis and regeneration (38). The property of quiescence or slow cycling in stem cells may facilitate resistance to many stressors (*e.g.*, oxidative stress, chemical compounds, and ionizing radiation) (57). Indeed, the quiescent HSC population is resistant to 5-fluorouracil (8).

Microenvironment of stem cells

Compelling evidence has demonstrated that the microenvironments of the HSCs, central nervous stem cells, intestinal epithelium, the hair follicle, and spermatogonial stem cells play a critical role in regulating the characterization of self-renewal and the differentiation of stem cells (38, 83). Two types of niches (osteoblastic and vascular) have been identified (39). The osteoblastic niche is characterized by hypoxia, which may facilitate the maintenance of quiescence of HSCs, while the vascular niche in the bone marrow is characterized by oxygenation that may facilitate the initiation of cycling of HSCs (129). Cipolleschi *et al.* showed that the colony-forming ability or reconstitution capacity of undifferentiated HSCs was retained after hypoxic culture compared with normoxic culture (26). Hypoxia may prevent the HSC side population (SP) from cycling (95). Similarly, in the hair follicle, the quiescent stem cells reside in the bulge region, while the active proliferating stem cells stay in the bulb region (87). These results suggest that the spatially distinct niches function to maintain stem cells in different states. The niches activate intracellular signaling pathways that induce the quiescent state of HSCs; receptor tyrosine kinase Tie2/Angiopoietin-1 (8), Mpl/Thrombopoietin (130), Wnt/β-catenin (72), and cell adhesion molecules (131) are involved. Additionally, the intracellular ROS level is a critical factor that regulates the quiescent status of HSCs (112). The quiescence of stem cells prevents telomere erosion, accumulation of DNA damage, and loss of the reconstitution capacity (48).

Intracellular ROS and their regulators in stem cells

The low number of stem cells makes it difficult to directly evaluate ROS levels and the redox regulatory mechanism in stem cells. However, interesting evidence has been obtained in HSCs. Similar to the low partial pressure of oxygen, low levels of ROS in niches are of importance for the stemness of HSCs (60). Jang and Sharkis (60) demonstrated that the DCF-DA^{low} (2,7-dichlorodihydrofluorescein diacetate, the redox-sensitive fluorescent probe) HSC population exhibited more traits of HSCs located within the osteoblastic niche, including high G_0 activity and high expressions of the calcium-sensing receptor, N-cadherin, Notch1, Bcrp, telomerase, and p21, which were pivotal in protecting the HSCs from exogenous and endogenous toxins. These markers were lower in the DCF-DA^{high} HSC population than in the DCF-DA^{low} population, and the HSC fraction with DCF-DA^{high} was "myeloid shifted" and behaved similar to aged HSCs (60). Several signaling molecules have been reported as being involved in the regulation of ROS in stem cells to maintain the quiescence of HSCs, and are discussed as follows.

Ataxia telangiectasia mutated

Ataxia telangiectasia mutated (ATM) is a critical regulator of the cell cycle and DNA damage repair, particularly for double-strand breaks. Cosentino *et al.* have shown that ATM reduces the ROS level by activating the glucose-6-phosphate dehydrogenase to promote NADPH production, an essential oxidant cofactor (29). Consistent with the results, ATM knockout mice displayed increased levels of ROS in many organs, and the mechanism may involve the reduced capacity of the major antioxidant enzymes, including catalase, GPX, SOD, and glutathione reductase (58), but the links between ATM and these genes are not clear. This increase of intracellular ROS appears to have deleterious effects (Fig. 2). For instance, the self-renewal capacity of HSCs was found to be decreased in $ATM^{-/-}$ mice compared with wild-type mice, and treatment with an antioxidant, *N*-acetyl L-cysteine (NAC), dramatically reversed this negative impact on the capability to reconstitute bone marrow hematopoietic cells. These results suggest that ATM may negatively regulate the ROS although the mechanism has not been thoroughly explained.

Phosphoinositide 3-kinase/Akt

One of the critical downstream signaling pathways of ATM is the phosphoinositide 3-kinase (PI3K)-Akt pathway. HSCs in Akt1- and Akt2-deficient mouse become quiescent and lose the capability of reconstitution (65). These HSCs also have reduced intracellular ROS content, indicating that ROS homeostasis is essential to sustain hematopoiesis and that Akt signaling is involved in the control of ROS levels as well (Fig. 2) (65). *In vitro* pretreatment of HSCs from Akt1- and Akt2-deficient mice with a low dosage of L-buthionine sulfoximine, an inhibitor of GSH synthesis, can rescue the decreased capability of colony formation (65). Additionally, Chen *et al.* (21) demonstrated that inhibition of the mammalian target of rapamycin (mTOR) pathway, a key regulator of cellular metabolism and a downstream target of Akt, can drive HSCs from quiescence into rapid cell cycling, with increased mitochondrial biogenesis and elevated levels of ROS.
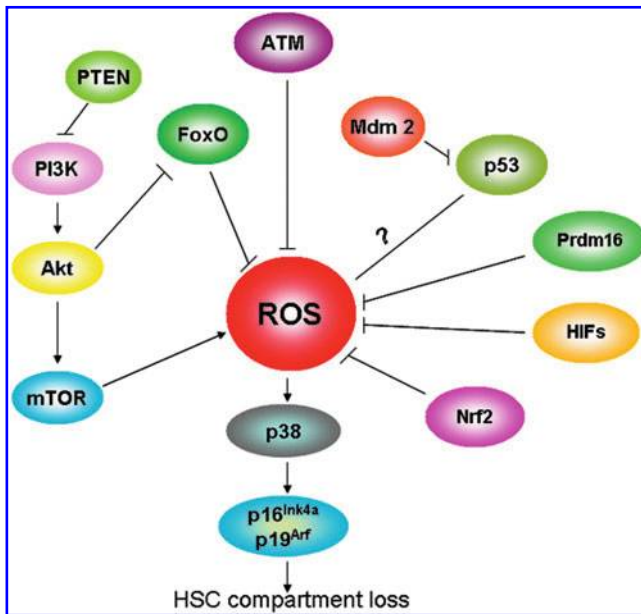
FIG. 2.   The signal pathways involved in ROS regulation in hematopoietic stem cells (HSCs). The activation of ataxia telangiectasia mutated (ATM) kinase, phosphoinositide 3-kinase (PI3K)/Akt, phosphorylates FoxO transcription factor 3 (FoxO3a), phosphatase and tensin homolog (PTEN), p53, PR domain-containing 16 (Prdm16), hypoxia inducible factor (HIF)-1α, p38 mitogen-activated protein kinase (MAPK), and nuclear factor erythroid-2-related factor 2 (Nrf2) negatively regulate ROS by up-regulating the antioxidant enzymes. PTEN can indirectly decrease ROS by negatively regulating the PI3K/Akt/mammalian target of rapamycin (mTOR), which can up-regulate the ROS levels. The increased ROS level can activate the p38MAPK, which can elevate the expression of the tumor suppressors p16^{Ink4a} and p19^{Arf}, resulting in HSC compartment loss. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

Phosphorylation of FoxO transcription factor 3a

FoxO, a family of the Forkhead transcription factors, is tightly regulated by PI3K/Akt. The phosphorylation of FoxO transcription factors 3 (FoxO3) by Akt leads to the FoxO3's association with 14-3-3 proteins and the FoxO3's retention in the cytoplasm (16). The sequestration of FoxO3 in the cytoplasm decreases the transcription activity of FoxO3-targeted genes such as SOD$_2$ and catalase, resulting in the elevation of ROS (Fig. 2). Accordingly, FoxO3a knockout mice exhibit increased levels of H$_2$O$_2$ with reduced activity of SOD$_2$ and catalase (82). In an independent study, HSCs in FoxO3a/ FoxO1/FoxO4 triple-knockout mice exhibit hyper-proliferation that leads to HSC pool exhaustion in the bone marrow, while treatment with NAC in these mice effectively improved the size of the HSC pool (112). Thus, FoxO proteins seem to be essential for maintaining the low level of intracellular ROS in HSCs.

Phosphatase and tensin homolog

Phosphatase and tensin homolog (PTEN), a negative regulator of PI3K/Akt, has the ability to promote cell differentiation and proliferation, leading to the HSC pool exhaustion (Fig. 2) (128). HSCs in conditional PTEN-deleted mice do not

exhibit increased ROS, and the reconstitution capacity of HSCs in these PTEN-negative mice cannot be rescued by NAC treatment (76). Obviously, further exploration about the relationship between the ROS and PTEN is still needed.

p53

Tumor suppressor p53, a transcription factor that transactivates a plethora of genes, is involved in regulating proliferation, differentiation, senescence, and apoptosis in response to DNA damage signals such as ROS (50). In an early report, p53 was shown to elevate ROS by activating ROS-inducing genes such as Pig1, Pig8, and Pig12 (35). In a conflicting report, p53 was shown to protect stem cells from ROS damage through up-regulation of GPX, p53-induce genes (PIGs) and down-regulation of nitric oxide synthase 2 (NOS2), cyclooxygenase 2 (COX2), and led to exhaustion of stem cells (Fig. 2) (1). Mdm2, an E3 ubiquitin ligase responsible for p53 degradation, is essential for cell survival. *In vivo* data demonstrate that *Mdm2* is necessary to control ROS-induced p53 levels for sustainable hematopoiesis later in life (1). By utilizing a rare p53 mutation (p53R172P, *i.e.*, arginine to proline at amino acid 172) that lacks apoptotic activity but with cell-cycle arrest activity, Abbas *et al.* (1) produced $Mdm2^{-/-}$ $p53^{515C}$ (encoding p53R172P) mice, which have a normal HSC count in fetal livers but a depleted HSC count in postnatal bone marrows. After birth, these mice had elevated ROS and expression of p53R172P, which leads to cell death in the hematopoietic compartment through activating p16^{Ink4a}. This postnatal loss of HSC could partially be antagonized with antioxidant treatment.

PR domain-containing 16

PR domain-containing 16 (Prdm16), a zinc-finger transcription factor and preferentially expressed by stem cells throughout the nervous and hematopoietic systems that control the development of blood cells (*e.g.*, leukocytes, platelets) and brown fat, has also been implicated in the regulation of ROS in HSCs (24). Prdm16 regulates leukemogenesis (24) and promotes neural stem/progenitor cell functions at least in part by promoting hepatocyte growth factor expression and inhibiting ROS generation. Prdm16 deficiency can lead to an increase in ROS levels, increased cell death, altered cell-cycle distribution, and depletion of stem cells (Fig. 2). Administration of anti-oxidant NAC to Prdm16-deficient mice could partially rescue the neural stem/progenitor cell dysfunction, indicating that Prdm16 could function as a modulator of oxidative stress in stem cell maintenance.

Hypoxia inducible factors

Hypoxia inducible factor-1α (HIF-1α) protein, a basic helix-loop-helix-PAS (Per-ARNT-Sim)-type transcriptional regulator under hypoxia, is involved in the maintenance of HSCs. ROS production is paradoxically increased under hypoxia, probably *via* electron leakage from ubisemiquinone to molecular oxygen (11). The depletion of molecular oxygen stabilizes and activates HIF-1α (20). It is known that HIF-1α activates vascular endothelial growth factor (VEGF), which has a strong correlation with cancer progression and metastasis, by directly binding to the VEGF promoter in response to hypoxia. An increased production of ROS was observed in HIF-1α$^{Δ/Δ}$ CD34$^-$LSK (Lineage$^-$ Sca-1$^+$c-Kit$^+$ population) cells, which may account for the loss of HSCs through

senescence-associated events (110). The HSCs in HIF-1α deficient mice lose their cell-cycle quiescence, and HSC numbers decreased in a p16^{Ink4a}/p19^{Arf}-dependent manner in various stress settings (110).

p38 mitogen-activated protein kinase

The mitogen-activated protein kinases (MAPKs) family, including extracellular signal-regulated protein kinases, c-Jun N-terminal kinases, and p38, plays an essential role in cell proliferation, differentiation, migration, and apoptosis by modulating gene transcription in the nucleus. The activation of p38MAPK through ROS-induced activation of apoptosis signal-regulated kinase 1 (ASK1) could lead to increased expression of p16^{Ink4a} and p19^{Arf}, which induced the loss of HSCs (Fig. 2) (59). The inhibition of p38MAPK rescued the ROS-induced defects in HSCs' repopulating capacity and maintenance of HSC quiescence, indicating that the ROS-p38MAPK pathway contributed to exhaustion of the stem cell population (69). These data support that p38MAPK activation can induce the loss of HSC self-renewal capacity (Fig. 2).

Nuclear factor erythroid-2-related factor 2

The nuclear factor erythroid-2-related factor 2 (Nrf2), a bZip transcription factor (18, 66), is also involved in the regulation of intracellular ROS. Once activated in response to a range of oxidative and electrophilic stimuli, Nrf2 mediates the expression of a spectrum of cytoprotective genes, including GSH, Trx, heme oxygenase-1, and the members of the glutathione-S-transferase family (111, 118). PhaseIIenzymes, NAD(P)H: quinine oxidoreductase (NQO1) is also regulated by Nrf2. Therefore, Nrf2 may help in maintaining the quiescence of HSCs by maintaining a reduced redox state (Fig. 2). However, Merchant et al. (81) found that the Nrf2$^{-/-}$ hematopoietic stem/progenitor cell compartment had increased rates of apoptosis without elevated levels of ROS, and the administration of NAC did not rescue the apoptosis of Nrf2$^{-/-}$ hematopoietic stem/progenitor cells; these findings suggest that Nrf2 may regulate hematopoiesis and HSCs survival in a ROS-independent manner. The precise mechanism of Nrf2 that maintains HSCs still needs to be elucidated.

In summary, ATM kinase, PI3K/Akt, FoxO3a, PTEN, p53, Prdm16, HIF-1α, p38MAPK, and Nrf2 are important for HSCs to maintain low levels of ROS, which may facilitate chemo/radio resistance and self-renewal of HSCs (Fig. 2). Mice deficiency in ATM, FoxO3a, Prdm16, or p53 exhibits elevated ROS levels in the HSC compartment that lead to a rapid exhaustion of HSCs. In vivo data support the fact that NAC restores the self-renewal capacity of HSCs in the aforementioned genes-deleted mice. These pieces of evidence support a causal effect of ROS on HSC demise.

Although the majority of the current literature supports the concept that low ROS facilitates the quiescent status of stem cells, one notable exception is that proliferative, self-renewing neural stem cells have a high level of ROS, and they are highly responsive to ROS stimulation with ROS serving as a second messenger that regulates normal cellular processes (75).

ROS and Cancer Cells

Cancer cells produce more ROS than normal cells do (109); ROS are involved in each stage of cancer development, including initiation, promotion, and progression (113). The increased levels of intracellular ROS can cause damage to DNA, lipids, and proteins, making cells more vulnerable to injury by further ROS insults induced by exogenous agents. This aspect has been exploited as an effective approach to selectively kill cancer cells without causing significant toxicity to normal cells (94, 113). The increase in intracellular ROS in cancer cells may involve a diversity of mechanisms. The intrinsic mechanism of intracellular ROS increase may result from the activation of oncogenes, inactivation of tumor suppressor genes, high metabolism, and mitochondrial dysfunction (113). It is common in cancers for metabolism to be very active under the drive of oncogenic signals, for example, constitutively active mutant Ras, Bcr-Abl, and c-Myc (54, 100, 115). Irani et al. (54) showed that the Ras-transformed NIH 3T3 fibroblasts produced O_2^- through the activation of NOX. Ras oncogenic signaling also suppressed the antioxidant molecule sestrin 1 (SESN1) (71). Transformation of the hematopoietic cell lines Ba/F3, 32Dc13, and MO7e with Bcr-Abl resulted in increased intracellular ROS compared with untransformed cells (100). The increase in ROS was directly due to Bcr-Abl, because it could be blocked by the small-molecule tyrosine kinase inhibitor ST1571. The loss-of-function mutation of Bcr-Abl such as Y177F failed to elevate the intracellular ROS levels in contrast with wild-type Bcr-Abl. Further, Bcr-Abl may increase ROS via activation of the PI3K/mTOR pathway, because inhibition of PI3K or mTOR attenuates the Bcr-Abl-induced ROS (70). Similarly, the activation of c-Myc could increase ROS without the induction of apoptosis, while the treatment with antioxidant NAC decreased the number of c-Myc-induced hMre11 signals and improved cell survival after c-Myc activation (115).

Tumor suppressor gene p53 decreases ROS by upregulating the transcription of antioxidant protein TIGAR (p53-induced glycolysis and apoptosis regulator), which inhibits glycolysis and SCO2 that can regulate the mitochondrial ETC (126). Splenocytes and thymocytes of p53$^{-/-}$ mice exhibit elevated ROS in comparison with wild-type mice.

Isocitrate dehydrogenase 1 (IDH1) and isocitrate dehydrogenase 2 (IDH2) are NADP+ dependent enzymes located in the cytoplasm and mitochondria, respectively, that convert isocitrate to α-ketoglutarate and generate NAD(P)H. Genome-wide sequencing studies also indicated somatic mutations in isocitrate dehydrogenase 1/2 (IDH1/2) in AML and central nervous system (CNS) tumors (119, 123). These mutations (e.g., R132 IDH1, R172 IDH2, and R140 IDH2) impair the enzyme's affinity for its substrate and dominantly inhibit wild-type IDH1/2 enzyme activity through the formation of catalytically inactive dimers (133), resulting in the depletion of NAD(P)H and GSH to an increase in ROS. Mutations in IDH1/2 also lead to the accumulation of 2-hydroxyglutarate, which is capable of elevating intracellular ROS levels to induce the differentiation of leukemic blasts (3). Additionally, IDH1/2 mutations decrease α-ketoglutarate to stabilize HIF-1α (78).

Mitochondrial DNA (mtDNA) mutations such as ND1, ND4L, and ND5 (subunits of complex I), cytochrome b (complex III), and COXI, COXII, and COXIII (complex IV) correlate with the increased levels of ROS in solid tumors and leukemia (17, 52, 55). The mutations of mtDNA are likely to impair electron transfer, leading to leakage of electrons and generation of O_2^- (15). Meanwhile, the active aerobic

glycolytic metabolism of cancer cells can also contribute to increase ROS (3).

The extrinsic mechanism of ROS increase may involve the abnormal microenvironment and therapeutic factors. Evidence shows that cancer cells have adaptation for persistently elevated ROS by (i) activating redox-sensitive transcription factors that upregulate ROS scavenging enzymes such as SODs and glutathione synthase, (ii) activating expression of the survival factors, and (iii) inhibiting cell death factors. These adaptive changes prevent the ROS level from reaching the cell-death threshold as well as from raising that threshold to promote cell survival and proliferation (113). Due to the increased ROS level, cancer cells may be more susceptive to oxidative damage induced by exogenous agents (including xenobiotics) and more reliant on the antioxidant system to sustain cell survival. For example, human leukemia cells with intrinsic oxidative stress are highly sensitive to ROS stress induced by 2-methoxyetradiol and arsenic trioxide (51, 134). Wu *et al.* (122) showed that diallyl disulfide, an oil soluble constituent of garlic (*Allium sativum*), could induce cell-cycle arrest at the G_2/M phase and apoptosis in human A549 lung cancer cells through the production of ROS, which could be completely abrogated by NAC. Trachootham *et al.* (114) showed that β-phenylethyl isothiocyanate could kill fludarabine-resistant chronic lymphoid leukemia cells through the depletion of glutathione and by raising the ROS, which mediates mitochondria damage and glutathionylation-dependent degradation of Mcl-1. Jin *et al.* (63) demonstrated that niclosamide, an anti-helminthic agent, could induce apoptosis by increasing the levels of ROS as well as by inhibiting nuclear factor-kappa B (NF-κB) in AML cells. The mechanism of antitumor activity of farnesyltransferase inhibitors was also reported to involve the generation of ROS (86, 104). Increasing ROS by compound piperlongumine kills cancer cells in a p53-independent manner (94).

Recently, microRNAs are also found to regulate ROS. MicroRNAs are a class of short non-coding RNAs that regulate post-transcriptional gene activity by degrading transcripts of target genes (53). Venkataraman *et al.* (116) found that ectopic expression of miR-128a in medulloblastoma cells increased

intracellular ROS (O_2^-) and inhibited cell growth. The regulatory effect of miR-128a on ROS may be exerted by targeting Bmi-1 (*via* binding 3′ UTR of Bmi-1), because co-transfection with Bmi-1 and miR-128a did not lead to an increase in ROS when compared with that of miR-128a transfection alone (116). However, the underlying mechanism that Bmi-1 increases ROS remains to be further explored.

ROS in CSCs

Concept of CSCs

CSCs only constitute a subset of the whole tumor cell population. They share the properties of self-renewal capacity and chemo-/radio resistance with normal stem cells. The study of leukemia stem cells has initiated this CSCs research area. A pioneering study showed that it is possible to use CD34$^+$/CD38$^-$ cell surface markers to separate cells into AML CSCs and non-CSCs populations that differ in their self-renewal capacity *in vitro* and tumorigenicity *in vivo* (14). Subsequent studies demonstrate that CSCs can be sorted and enriched by cell surface markers in various cancer types (Table 1 and Fig. 3). For instance, ESA$^+$/CD44$^+$/CD24$^-$/ Lin$^-$ breast cancer cells are capable of generating the phenotypic heterogeneity recapitulating the initial tumor of a patient, and this breast cancer cell sub-population possesses high tumorigenicity and treatment resistance similar to stem cells (14, 78, 90). In the CNS, CD133 is believed to be the marker for CSCs in different types of brain tumor (105). The subpopulation of CD133$^+$ tumor cells is enriched after radiation in gliomas (9). In the absence of known cell surface markers, the SP in flow cytometry seems to be a useful method for isolating the CSCs (11, 12, 14). CSCs are capable of pumping fluorescent dye out of the cells because of the enhanced expression of an ATP-binding cassette (ABC) transporter, ABCG2. Therefore, the cancer cells in the SP fraction possess the properties of stem cells (73). Interestingly, Radovanovic's research group isolated a subpopulation from human glioma tissues and cultures based on intrinsic autofluorescence properties and distinctive cell morphology, and this subpopulation preferentially expressed embryonic

TABLE 1. CELL SURFACE PHENOTYPE OF CANCER STEM CELLS IDENTIFIED IN HUMAN CANCERS

Tumor type	CSC phenotype	References
AML	CD34$^+$/CD38$^-$, CD44$^+$,CD34$^+$/CD123$^+$, CD47$^+$	(14, 61, 62, 64, 79)
ALL	CD34$^+$/CD10$^-$, CD34$^+$/CD19$^-$;	(30, 74)
Breast cancers	CD44$^+$/ESA$^+$/CD24$^-$/Lin$^-$, ALDH1^{low}	(6, 41)
Brain tumors	CD133$^+$, CD15$^+$	(105, 106)
Melanoma	ABCB5$^+$, CD271$^+$, JARID1B$^+$, CD133$^+$, CD20$^+$	(13, 37, 93, 99, 101)
Colorectal cancers	CD44$^+$/EpCAM$^+$,CD133$^+$, ALDH1^{high}, Lgr-5$^+$	(10, 32, 49, 85, 97)
Lung cancers	CD133$^+$, CD133$^+$/EpCAM$^+$	(36, 67)
Osteosarcomas	CD44$^+$/CD105$^+$/Stro1$^+$	(40)
Head and neck squamous cell carcinomas	CD44$^+$/ALDH^{high}	(23, 92)
Liver cancers	CD13$^+$,CD90$^+$/CD44$^-$, CD133$^+$, CD24$^+$	(68, 77, 108, 124)
Pancreatic cancers	CD44$^+$/CD24$^+$/ESA$^+$, CD133$^+$	(47)
Prostatic cancers	CD44$^+$/Integrin $\alpha_2\beta_1^{high}$/CD133$^+$	(28)
Skin squamous cell carcinomas	CD34$^+$, Integrin $\alpha_5\beta_1^{high}$	(80, 103)
Ovarian cancers	CD44$^+$/CD117$^+$, CD133$^+$	(31, 132)
Bladder cancers	CD44$^+$	(19)

ALDH1, aldehyde dehydrogenase-1; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; EpCAM, epithelial cell adhesion molecule; ESA, epithelial special antigen; JARID1B, the H3K4 demethylase; Lgr-5, leucine-rich-repeat containing G-protein-coupled receptor 5.
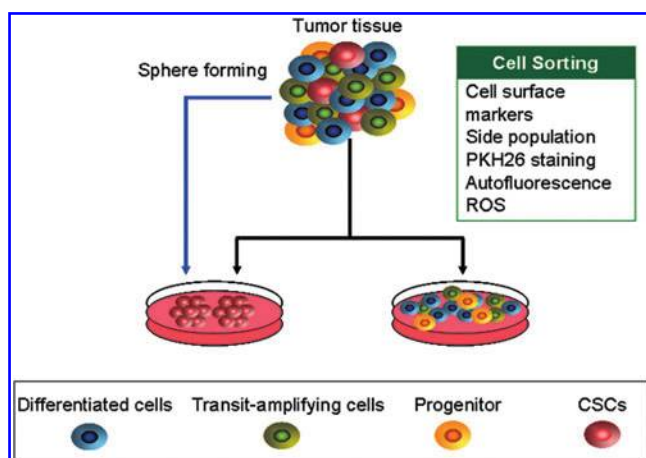
FIG. 3. **Isolation of cancer stem cells (CSCs).** CSCs can be sorted out on the basis of antigens, functional markers, side population, PKH26 dye intensity, autofluorescence, and ROS levels using flow cytometry (fluorescence-activated cell sorting). (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

stem cell markers, such as octamer-binding transcription factor 4 (Oct4), Nanog, and sRY (sex determining region Y)-box 2 (Sox2), and have the properties of CSCs (27). Of note, PKH26, a lipophilic dye, is becoming a popular tool for isolating CSCs in some types of cancer (25, 88). CSCs can retain PKH26 dye on the basis of asymmetric division. Therefore, CSCs can be isolated by FACS as the PKH26^{high} population (Fig. 3).

It is clear that normal stem cells are pluripotent, but how about the differentiation capacity of CSCs? Recent studies showed that CSCs have endothelial and adipocytic lineage potential (5, 91, 98). De Maria *et al.* showed that a majority of endothelial cells in glioblastoma carry the same mutation as the cancer cells (98). This result suggests that CSCs may at least contribute to the origin of vascular endothelium in the tumor. Therefore, CSCs seem to be capable of trans-differentiating into vascular cells and forming vasculogenic mimicry (7).

On the other hand, increasing evidence indicates that CSCs have an enhanced capacity to initiate and sustain tumor growth, which is important for the progression and relapse of malignant tumors (117). Previous studies speculated that CSCs may differentiate into cancer cells in various stages of maturation by a unidirectional process. However, recent studies tell us another interesting story: the relationship between CSCs and non-CSCs may be bi-directional, and non-CSCs may be reprogrammed into CSCs (42). Furthermore, the kinetic rate of both directions (CSCs to non-CSCs or non-CSCs to CSCs) is not low. This equilibrium may be shifted in either one direction or another based on the contextual signals of the tumor microenvironment (42). Therefore, inter-conversion between the CSCs and non-CSCs compartments seems to be one reason that can explain the variation in the frequency of CSCs in different stages of disease and various types of cancer.

Hypoxia in the solid tumor microenvironment has been shown to be a regulator that maintains the stem-like phenotype of colorectal cancer cell line-derived CSCs and prevents differentiation of enterocytes and goblet cells by regulating CDX1 and Notch1 (127). Given that hypoxia is capable of inducing ROS and the stabilization of HIF1α, more work is needed to elucidate whether there is a linkage between ROS-HIF-1α and CDX1/Notch1 that controls the switch between stemness and differentiation in mature cancer cells.

Regulation of ROS in CSCs

In comparison with normal stem cells or cancer cells as a whole population, relatively little is known in CSCs about ROS levels. Similar to normal stem cells such as HSCs, CSCs also show lower intracellular ROS contents than non-CSCs, which may be due to the increased expression of free radical scavenging systems (34, 125).

CD44, an adhesion molecule, is expressed in CSCs of various types of cancers. Its variant isoform CD44v as a result of alternative mRNA splicing with insertions in the membrane-proximal extracellular region can protect gastric cancer CSCs from high levels of ROS in the tumor microenvironment (6, 28,

FIG. 4. **CD44v enhancement of ROS defense by interacting and stabilizing xCT.** The CD44v molecule interacts with xCT, a light-chain subunit of cystine-glutamate exchange transporter at the plasma membrane. xCT elevates cystine uptake that facilitates intracellular reduced glutathione (GSH) synthesis, and reduces the ROS levels and ROS-dependent activation of p38MAPK. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).
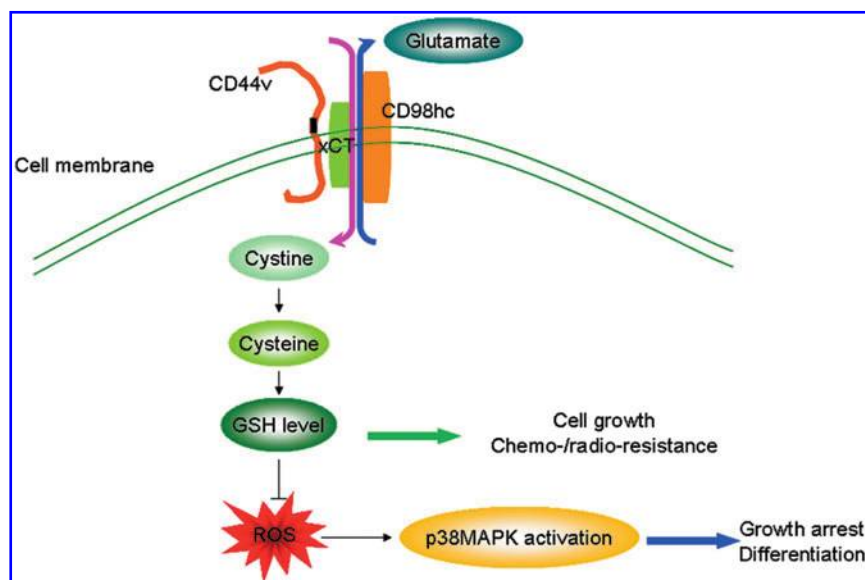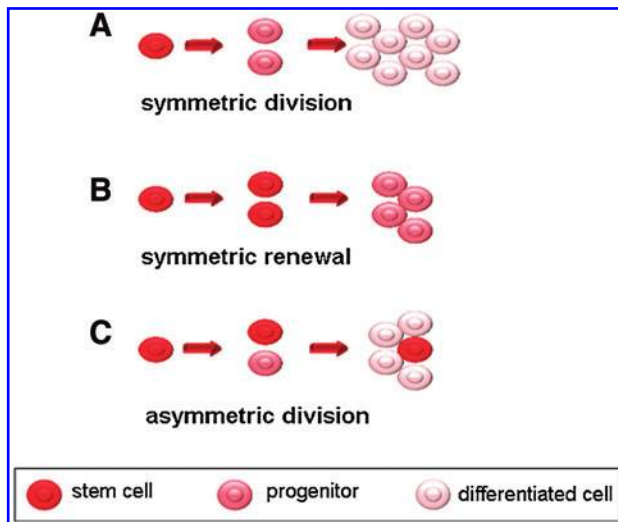
FIG. 5. **Models for cell division.** Stem cells may be divided in the following three possible ways: **A**, Symmetric division: a stem cell divides into two progenitor cells; **B**, Symmetric renewal: a stem cell divides into two stem cells. **C**, Asymmetric division: a stem cell divides into one progenitor cell and one stem cell. (To see this illustration in color the reader is referred to the web version of this article at www .liebertonline.com/ars).

32). Ishimoto *et al.* (56) demonstrated that CD44v in gastrointestinal CSCs can enhance the intracellular GSH levels by stabilizing xCT, a light-chain subunit of a glutamate-cystine exchange transporter located at the plasma membrane. The function of xCT at the cell surface is to increase the uptake of cystine that is a substrate for GSH synthesis, and, therefore, xCT is critical for controlling the intracellular redox status (Fig. 4). The ablation of CD44v inhibits the activity of xCT, and results in increased ROS and ROS-dependent p38MAPK activation. This is supported by the observation that the ROS levels detected by DCFH-DA staining in CD44^{low} or CD44$^-$ tumor cells are much higher than those in CD44^{high} tumor cells. Moreover, the silencing of CD44 by RNA interference

leads to increased ROS levels, and the administration of NAC could reverse the oxidative stress phenotype in CD44$^-$ cells. Thus, CD44v-targeted therapy that impairs ROS defense may provide a potential approach of killing CSCs.

Further, CD44$^+$/CD24$^-$ breast CSCs have been found to exhibit an enhanced ROS defense system by over-expressing antioxidant enzyme genes and less DNA damage when compared with non-CSCs (34). Lower levels of basal and radiation-induced ROS in CD44$^+$/CD24$^-$ breast CSCs have been associated with the tumorigenicity and resistance to radiation (34). Given a link between the management of ROS by CSCs and enhanced tumor radio resistance, a pharmacological increase in ROS in CSCs may decrease their clonogenicity and result in radio sensitization.

CSCs molecule CD13 negatively regulates ROS with a resultant increase of stemness in liver CSCs. Kim *et al.* (68) showed that increased CD13 expression could reduce ROS and promote the survival of liver CSCs *via* a transforming growth factor-beta induced epithelial mesenchymal transition-like process. CD13 is also associated with increased ROS scavenger capacity in human liver CSCs. Gclm, encoding the glutamate-cysteine ligase that catalyzes the rate-limiting step in the synthesis of GSH, was over-expressed in the CD13$^+$/CD90$^-$ and the CD13$^+$ fractions of cells (HuH7 and PLC/PRF/5), and these cell fractions have lower concentrations of ROS than the CD13$^-$ fraction. Due to the suppression of CD13 with the CD13-neutralizing antibody (clone WM15) or ubenimex, a compound that specifically blocks CD13 by antagonizing the zinc-binding site of the aminopeptidase N domain, the ROS concentration was significantly increased in CD13$^+$ cells and reached the level of ROS observed in the CD13$^-$ cells (46). Moreover, administration with ubenimex inhibited the self-renewal and the tumor-initiation ability of CD13$^+$ cells in mice xenografted in a CD13$^+$ cell-enriched fraction (46). Targeting CD13 with reagents such as anti-CD13 neutralizing antibody and ubenimex may offer a therapeutic approach for CSCs elimination.

Here, we raise questions for discussion. First, why do CSCs show lower ROS levels than non-CSCs? Second, why do cancer cells keep a subset of ROS^{low} cells in the whole cell
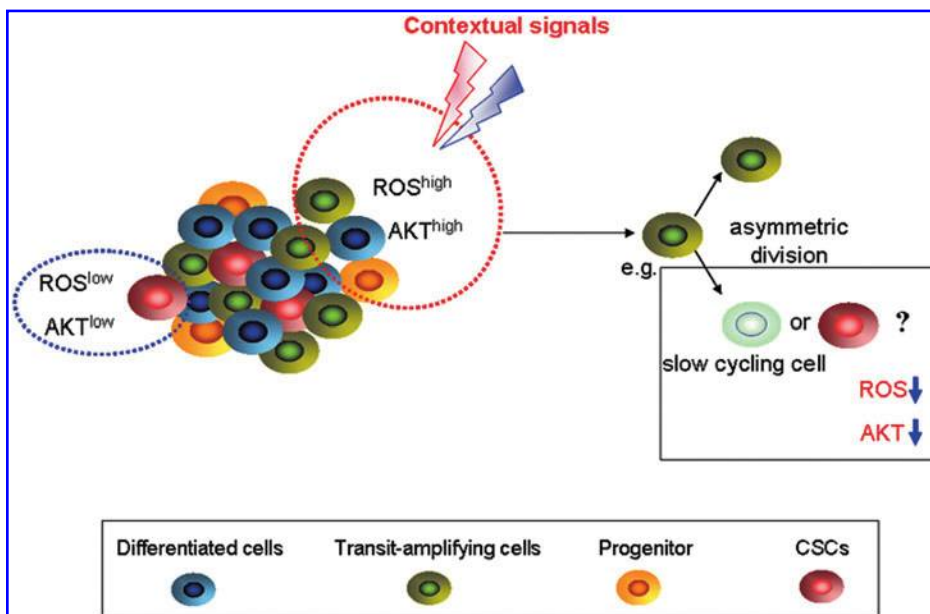

FIG. 6. **Rapidly proliferating cancer cells produce ROS^{low} cells by asymmetric division.** ROS^{low} cancer cells with low Akt activity are predominantly seen in the G_1/G_0 of the cell cycle compared with actively cycling ROS^{high} cells. The stimulators that modulate Akt signaling can shift the dynamic between symmetric and asymmetric division. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline .com/ars).

population? The evidence until now is rather limited. Ramaswamy *et al.* found that rapidly proliferating breast cancer cells can produce "G_0-like" progeny by asymmetric division (Fig. 5) (33). The cells in the G_0 phase showed lower intracellular ROS contents and decreased Akt activation. Inhibition of the Akt signaling pathway in proliferating cancer cells resulted in an increased ratio of asymmetric division and produced large amounts of ROS^{low} cells with slow growth (Fig. 6). The stimulators that modulate Akt signaling can shift the dynamics between symmetric and asymmetric division, similar to the equilibrium between CSCs and non-CSCs. We hypothesize here that producing the cells with stem cell characteristics and ROS^{low} cells during proliferation and passage is the protective and "talented" behavior of cancer cells. Cancer should protect a subset of "tumor seeds" from DNA damage that facilitates tumor propagation. Additionally, ROS^{low} cells could be enriched after chemotherapy in breast cancer patients (34), indicating that ROS^{low} cells should be targeted during cancer therapy.

In addition, is exogenous ROS elevation capable of killing CSCs similar to killing non-CSC cancer cells? The answer may be optimistic. Niclosamide, a potent agent that increases ROS levels, can selectively kill CD34$^+$/CD38$^-$ AML stem cells while having minimal cytotoxicity against the progenitor cells in normal bone marrow cells from healthy individuals (63). The antineoplastic mechanism of niclosamide may also involve inactivation of the NF-κB pathway (63). Consistent with this finding, Guzman *et al.* showed that parthenolide (a natural product) can also induce apoptosis in the CSCs of AML and CML patients at least partially *via* an increase in ROS (44). The tumor suppressor gene PML (promyelocytic leukemia), which is involved in the t(15;17) chromosomal translocation of acute promyelocytic leukemia, has been demonstrated to be an important factor that maintains the quiescent state of CML CSCs (57). Arsenic trioxide (AS$_2$O$_3$), which can lead to PML degradation and increase ROS, is presumed to eliminate CML stem cells (57). To date, the mechanisms that ROS kill CSCs are elusive. However, at least, high levels of ROS in niches may trigger differentiation (3).

Conclusions

Exogenously increased ROS have been shown to selectively kill cancer cells. Low levels of ROS are associated with the stemness of stem cells and CSCs. However, the underlying mechanism for decreased ROS has not been adequately understood. Intensive research for ROS in CSCs is desperately needed. Oxidative stress exists in cancer cells, and exogenous agents may further increase ROS effectively and selectively kill cancer cells. It will be of interest to elucidate whether increasing ROS levels represent an efficacious strategy to eradicate CSCs.

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Author Disclosure Statement

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Abbreviations Used

ABC = ATP-binding cassette
ABCB5 = a novel human ABC transporter
ALDH1 = aldehyde dehydrogenase-1
ALL = acute lymphoblastic leukemia
AML = acute myeloid leukemia
ASK1 = apoptosis signal-regulatory kinase 1
ATM = ataxia telangiectasia mutated
COX2 = cyclooxygenase 2
CSCs = cancer stem cells

DCF-DA = 2,7-dichlorodihydrofluorescein diacetate
EpCAM = epithelial cell adhesion molecule
ESA = epithelial- special antigen
ETC = electron transport chain
FoxO3 = phosphorylates FoxO transcription factor 3
GPX = glutathione peroxidase
GSH = glutathione
GSSG = oxidized glutathione
HIF-1α = hypoxia inducible factor-1α
HO$^\bullet$ = hydroxyl free radical
H_2O_2 = hydrogen peroxide
HOCl = hypochlorous acid
HSCs = hematopoietic stem cells
IDH = isocitrate dehydrogenase
JARID1B = the H3K4 demethylase
Lgr-5 = leucine-rich-repeat containing G-protein-coupled receptor 5
MAPK = mitogen-activated protein kinase
MPD = myeloperoxidase
mTOR = mammalian target of rapamycin
NAC = *N*-acetyl-cysteine
NF-κB = nuclear factor-kappa B
NO$^\bullet$ = nitric oxide
NOD/SCID = nonobese diabetic severe combined immunodeficiency disease
NOS = nitric oxide synthase
NOX = NAD(P)H oxidase complex
Nrf2 = nuclear factor erythroid-2-related factor 2
O_2^- = superoxide
Oct4 = octamer-binding transcription factor 4
ONOO$^-$ = peroxynitrite
PI3K = phosphoinositide 3-kinase
PIGs = p53-induced genes
PML = promyelocytic leukemia gene
Prdm 16 = PR domain-containing 16
PTEN = phosphatase and tensin homolog
RNS = reactive nitrogen species
ROS = reactive oxygen species
SOD = superoxide dismutase
Sox2 = sRY (sex determining region Y)-box 2
SP = side population
VEGF = vascular endothelial growth factor

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